

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)	MAIL STOP
Anthony Jevnikar et al.)	APPEAL BRIEF - PATENTS
Application No.: 10/005,073)	Group Art Unit: 1644
Filed: December 7, 2001)	Examiner: Gerald R Ewoldt
For: METHODS AND PRODUCTS FOR)	Appeal No.: _____
CONTROLLING THE IMMUNE)	
RESPONSES IN MAMMALS)	
)	
)	

APPEAL BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This appeal is from the decision of the Primary Examiner dated April 14, 2009 finally rejecting claims 52, 59-61, 63, 69-91, 95 and 102, which are reproduced as the Claims Appendix of this brief.

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The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800.

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I. Real Party in Interest

The present application is assigned to London Health Sciences Centre. London Health Sciences Centre is the real party in interest, and is the owner of the entire right and interest in Application No. 10/005,073.

II. Related Appeals and Interferences

The Appellants' legal representative, or assignee, does not know of any other appeal or interferences which will affect or be directly affected by or have bearing on the Board's decision in the pending appeal.

However, it is noted that claims 52, 59, and 60 have been rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claims 17-20, 34, and 53 of U.S. Patent Application No. 11/815,359. *See* OFFICE ACTION DATED APRIL 14, 2009, at 12. The rejection is necessarily provisional in nature. The claims of the '359 application have not issued at the time that this appeal is being filed. The claims in the '359 application may be allowed, amended, or the facts underlying this issue may change before this appeal is taken up by the Board. Applicants appeal the rejection as currently stated.

III. Status of Claims

Claims 1-51 have been canceled.

Claims 52-102 are pending in the application.

Claims 53-58, 62, 64-68, 92-94, and 96-101 have been withdrawn from consideration pursuant to a restriction requirement.

Claims 52, 59-61, 63, 69-91, 95 and 102 stand rejected and are the subject of this appeal.

IV. Status of Amendments

No amendments have been submitted since the final decision of the Examiner.

V. Summary of Claimed Subject Matter

There are five (5) independent claims on appeal, claims 52, 63, 84, 88, and 102. A concise explanation of the subject matter defined in each of the independent claims involved in the appeal, referring to exemplary description in the specification by page and line number is provided below:

A. Claim 52

Claim 52 is reproduced here with citations to exemplary disclosure in the specification by page and line in bold curly brackets. Claim 52 is directed to: A method for suppressing or reducing the immune response of a mammal {**page 7, lines 2-13**} to an antigen {**page 7, lines 2-13, page 9, lines 5-8; page 10, lines 10-25, page 10, line 34 to page 11, line 11**} comprising: orally or enterally administering to the mammal an effective immune suppressive dose {**page 14, lines 17-23**} of a plant tissue { **page 7, lines 21-29**} or a partially purified plant tissue extract {**page 15, lines 17-21**} containing said antigen or an immunosuppressive fragment {**page 7, lines 30-35**} thereof, said plant tissue or partially purified plant tissue extract being obtained from a transgenic plant expressing said antigen or immunosuppressive fragment thereof {**page 11, line 12 to page 14, line 23**}.

This claim is directed to a method of suppressing or reducing the immune response of a mammal to an antigen. Suppressing or reducing the immune response of a mammal to an antigen means that the mammal's tolerance to that antigen is increased. This is of benefit in, for example, the treatment of autoimmune disease and in preventing graft rejection following transplantation. See page 7, lines 2-13.

The “antigen” in this claim may be any immune response provoking protein or polypeptide, such as an autoantigen or a transplantation antigen. See page 7, lines 2-13. Examples of such antigens include, but are not limited to, the Major Histocompatibility Complex (MHC) class I and II molecules; myelin basic protein (MBP); glutamic acid dehydrogenase (GAD); islet cell-specific antigen (ICA); thyroglobulin; collagen; insulin; heat shock protein; islet granule associated antigen; and the islet derived glycoprotein p69. See page 9, lines 5-8; page 10, lines 10-25, page 10, line 34 to page 11, line 11.

An “effective immune suppressive dose” is a dose sufficient to suppress or reduce the immune system of the mammal to a specific antigen, thereby inducing tolerance to that antigen. In one example, an effective dose comprises around 30 grams of leaf material, when GAD protein comprises approximately 0.4% of total soluble leaf protein. See page 14, lines 17-23.

Plant materials within the scope of the presently claimed invention are described on page 7, lines 21-29 of the description as including plant tissues or plant parts containing the desired protein (i.e., antigen). The plant tissue may be, for example, leaves or tubers, which may be consumed raw or after minimal culinary preparation. Plant preparations or extracts may comprise total plant protein or purified or partially purified preparations of the transgenic protein of interest. In particular, if a non-edible plant is used for production of mammalian antigens, the antigens may be extracted from the plant tissue and purified as required by standard methods before oral or enteral administration. See page 15, lines 17-21. The plant tissue or partially purified plant tissue extract is obtained from a transgenic plant expressing the antigen or immunosuppressive fragment thereof. See page 11, line 12 to page 14, line 23.

An “immunosuppressive fragment” of an antigen is a portion of the amino acid sequence of the antigen which is capable, on oral or enteral administration to a mammal, of

inducing tolerance or suppressing the immune response of the mammal to the antigen. See page 7, lines 30-35.

Independent claim 52 is argued together with dependent claims 59, 60, and 61.

B. Claim 63

Claim 63 is reproduced here with citations to exemplary disclosure in the specification by page and line in bold curly brackets. Claim 63 is directed to: A pharmaceutical composition for suppressing or reducing the immune response of a mammal {**page 7, lines 2-13**} to an antigen { **page 7, lines 2-13, page 9, lines 5-8; page 10, lines 10-25, page 10, line 34 to page 11, line 11**} comprising: an oral or enteral dosage form {**page 7, lines 21-29**} comprising an effective immunosuppressive dose {**page 14, lines 17-23**} of a plant tissue {**page 7, lines 21-29**} or partially purified plant tissue extract {**page 15, lines 17-21**} containing said antigen or an immunosuppressive fragment thereof {**page 7, lines 30-35**} and a pharmaceutically acceptable carrier, said plant tissue or partially purified plant tissue extract being obtained from a transgenic plant expressing said antigen or immunosuppressive fragment thereof {**page 11, line 12 to page 14, line 23**}.

Claim 63 is directed to a pharmaceutical composition for suppressing or reducing the immune response of a mammal to an antigen. As was discussed above, suppressing or reducing the immune response of a mammal to an antigen means that the mammal's tolerance to that antigen is increased. This is of benefit in, for example, the treatment of autoimmune disease and in preventing graft rejection following transplantation. See page 7, lines 2-13.

The "antigen" in this claim may be any immune response provoking protein or polypeptide, such as an autoantigen or a transplantation antigen. See page 7, lines 2-13. Examples of such antigens include, but are not limited to, the Major Histocompatibility Complex (MHC) class I and II molecules; myelin basic protein (MBP); glutamic acid dehydrogenase (GAD); islet cell-specific antigen (ICA); thyroglobulin; collagen; insulin; heat

shock protein; islet granule associated antigen; and the islet derived glycoprotein p69. See page 9, lines 5-8; page 10, lines 10-25, page 10, line 34 to page 11, line 11.

An “effective immune suppressive dose” is a dose sufficient to suppress or reduce the immune system of the mammal to a specific antigen, thereby inducing tolerance to that antigen. In one example, an effective dose comprises around 30 grams of leaf material, when GAD protein comprises approximately 0.4% of total soluble leaf protein. See page 14, lines 17-23.

Plant materials within the scope of the presently claimed invention are described on page 7, lines 21-29 of the description as including plant tissues or plant parts containing the desired protein (i.e., antigen). The plant tissue may be, for example, leaves or tubers, which may be consumed raw or after minimal culinary preparation. Plant preparations or extracts may comprise total plant protein or purified or partially purified preparations of the transgenic protein of interest. The plant tissue or partially purified plant tissue extract is obtained from a transgenic plant expressing the antigen or immunosuppressive fragment thereof.

An “immunosuppressive fragment” of an antigen is a portion of the amino acid sequence of the antigen which is capable, on oral or enteral administration to a mammal, of inducing tolerance or suppressing the immune response of the mammal to the antigen. See page 7, lines 30-35.

Independent claim 63 is argued together with dependent claims 69-83.

C. Claim 84

Claim 84 is reproduced here with citations to exemplary disclosure in the specification by page and line in bold curly brackets. Claim 84 is directed to: A method for suppressing the rejection of engrafted donor tissue in a recipient mammal **{page 7, lines 8-11}** comprising orally or enterally administering to the mammal an effective

immunosuppressive dose **{page 14, lines 17-23}** of a plant tissue or a partially purified plant tissue extract containing a transplantation antigen of said donor tissue **{page 7, lines 2-13, page 9, lines 5-8; page 10, line 34 to page 11, line 11}** or an immunosuppressive fragment thereof **{page 7, lines 30-35}**, said plant tissue or partially purified plant tissue extract being obtained from a transgenic plant expressing said transplantation antigen or immunosuppressive fragment thereof **{page 11, line 12 to page 14, line 23}**.

Claim 84 is directed to a method for suppressing the rejection of engrafted donor tissue in a recipient mammal. This claim is similar to claim 52 and more specifically defines certain features of the claimed invention. For example, the specific immune response suppressed by the method of claim 84 is the “rejection of engrafted donor tissue” and the antigen is a “transplantation antigen”. Transplantation antigens provoke an immune response against engrafted tissue, leading to graft rejection. See page 7, lines 8-11.

Claim 84 is argued together with dependent claims 85-87.

D. Claim 88

Claim 88 recites A transgenic plant **{page 3, line 34 to page 4, line 2, page 11, line 12 to page 14, line 23}** comprising a plant expressing a recombinant mammalian transplantation antigen **{page 7, lines 2-13, page 9, lines 5-8; page 10, line 34 to page 11, line 14}**.

A “transgenic plant” is a plant that has had inserted into its genome one or more DNA sequences encoding mammalian antigens. The transgenic plants express the inserted DNA sequences and produce the antigens encoded therein. See page 3, line 34 to page 4, line 2.

A “recombinant mammalian transplantation antigen” is an antigen that may be introduced into and expressed by suitable plant hosts. See page 11, lines 12-14. A cDNA sequence coding for the selected mammalian antigen is inserted into a DNA construct under control of a promoter functional in plant cells and in proper reading with a transcription

termination sequence functional in plant cells, to provide for expression of the mammalian DNA in plant tissue. See page 11, lines 15-22.

Independent claim 88 is argued together with dependent claims 89-91 and 95.

E. Claim 102

Claim 102 is reproduced here with citations to exemplary disclosure in the specification by page and line in bold curly brackets. Claim 102 is directed to: A method for suppressing or reducing the immune response of a mammal to an MHC Class II protein {**page 7, lines 8-11**} comprising: orally or enterally administering to the mammal an effective amount of a plant tissue or a partially purified plant tissue extract obtained from a transgenic plant expressing an MHC Class II protein {**page 8, lines 21-24**} or an immunosuppressive fragment thereof {**page 7, lines 30-35**}.

This claim is directed to a method for suppressing or reducing the immune response of a mammal to an MHC Class II protein. This claim is similar to claim 52 and more specifically defines certain features of the claimed invention. For example, the antigen is an “MHC Class II protein”. MHC Class II proteins are integral transmembrane proteins encoded by polymorphic genes of the MHC and are members of the immunoglobulin superfamily. See page 8, lines 21-24. Both transplant rejection and autoimmune diseases, for example, may be reduced or suppressed by induction of tolerance to MHC Class II proteins by administration of plant material from suitably transformed plants. See page 9, lines 5-8 and page 10, lines 10-14.

Although specific exemplary citations for elements of the claims have been provided above as required by 37 C.F.R. § 41.37, the application is replete with supporting material for all of the presently pending claims, both in the originally filed text and in the originally filed drawings. The exemplary citations to the specification should not be construed as limiting the claims.

VI. Grounds of Rejection to be Reviewed on Appeal

The grounds of rejection to be reviewed on appeal are as set forth in the Office Action of April 14, 2009:

A. Rejection under 35 U.S.C. § 112, first paragraph, enablement

“Claims 52, 59-61, 63, 69-91, 95, and . . . 102 stand/are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.” *See* OFFICE ACTION DATED APRIL 14, 2009, at 2.

B. Rejection under 35 U.S.C. § 112, first paragraph, written description

“Claims 52, 59-61, 63, 69-76, 78-91, 95, and 102 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in relevant art that the inventor, at the time the application was filed, had possession of the claimed invention.” *See* OFFICE ACTION DATED APRIL 14, 2009, at 13.

C. Rejection under 35 U.S.C. § 103

“Claims 52, 59-61, 63, 69-76, 78-91, 95, and 102 stand/are rejected under 35 U.S.C. 103(a) as being unpatentable over WO 92/07581 (1992, IDS) in view of U.S. Patent No. 5,484,719 (IDS).” *See* OFFICE ACTION DATED APRIL 14, 2009, at 9.

D. Rejection under the judicially created doctrine of obviousness-type double patenting

“Claims 52, 59, and 60 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claims 17-20, 34, and 53 of U.S. Patent Application No. 11/815,359.” *See* OFFICE ACTION DATED APRIL 14, 2009, at 12.

VII. Argument

A brief overview of the invention may assist in understanding the issues on appeal. As noted in the *Background of the Invention* section of the present application, the related art in the field of systemic immunosuppressive therapy (i.e., by administration of immunosuppressive drugs) suffers from drawbacks such as increased rates of infection, malignancy, and numerous side effects. *See* SPECIFICATION, at 1, lines 13-15. Therefore, groups have pursued the induction of oral tolerance as a preferred method of immunosuppressive therapy. However, there are several problems associated with the prior art with respect to the induction of oral tolerance: “Firstly, the complexity of foreign peptide presentation in transplantation makes it difficult to screen peptide sequences suitable for induction of tolerance. Secondly, the induction of oral tolerance to antigens is dose dependent and insufficient oral antigen may prime gut lymphocytes and cause the opposite and undesired effect of sensitisation.... Thirdly, the nature of the peptide itself may cause increased rather than reduced immune responsiveness.” *See* SPECIFICATION, at 2, lines 16-28. It is advantageous to use whole antigen proteins to induce oral tolerance, as there is a greater array of potentially tolerance-inducing peptides presented to the immune system. However, when complex antigens such as MHC proteins or other transplantation antigens are used as intact proteins, it is difficult to obtain these proteins in sufficient quantities by prior art methods such as *in vitro* synthesis. *See* SPECIFICATION, at 2, lines 29-35.

The presently claimed invention overcomes at least the above-referenced drawbacks by expressing appropriate mammalian antigens, for example transplantation antigens or autoantigens, in plants. These plants, or materials derived from these plants, are administered to a mammal to produce oral tolerance to the expressed mammalian antigens in order to control or suppress the immune system. *See* SPECIFICATION, at 3, lines 1-8. The presently claimed invention “provides for producing immune-response provoking proteins or

polypeptides in quantities suitable for the induction of oral tolerance in mammals, including humans, by transforming plants to produce the desired proteins or polypeptides or immunosuppressive, tolerance inducing fragments or derivatives of these proteins or polypeptides.” *See* SPECIFICATION, at 7, lines 2-8.

A. Claim Rejections Under 35 U.S.C. §112, First Paragraph, Enablement

Claims 52, 59-61, 63, 69-91, 95, and . . . 102 stand rejected under 35 U.S.C. 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.” *See* OFFICE ACTION DATED APRIL 14, 2009, at 2.

The first paragraph of 35 U.S.C. § 112 requires that the specification enable any person skilled in the art to which it pertains to make and use the claimed invention without “undue experimentation.” *See In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed.Cir. 1988). That some experimentation may be required is not fatal; the issue is whether the amount of experimentation required is “undue.” *Id.* at 736-37, 8 USPQ2d at 1404. The Examiner bears the burden of establishing a prima facie case by presenting evidence and explanation which would lead a person of ordinary skill to doubt the enablement of the claimed invention.

When rejecting a claim under the enablement requirement of section 112, the PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application; this includes, of course, providing sufficient reasons for doubting any assertions in the specification as to the scope of enablement.

In re Wright, 27 USPQ2d 1510 (Fed. Cir. 1993)

[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the

first paragraph of Section 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

In re Marzocchi, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971).

The Examiner has asserted that the specification does not provide a demonstration of oral induction of tolerance. OFFICE ACTION DATED APRIL 14, 2009, at 6. Examples are not required to satisfy section 112, first paragraph. *In re Strahilevitz*, 212 USPQ 561 (C.C.P.A. 1982)(citing, e.g., *In re Stephens*, 529 F.2d 1343, 188 USPQ 659 (CCPA 1976); *In re Borkowski*, 57 CCPA 946, 422 F.2d 904, 164 USPQ 642 (1970); *In re Gay*, 50 CCPA 725, 309 F.2d 769, 135 USPQ 311 (1962)). Thus, the court held that the examiner's statement that the “nearly universal applicability” alleged for the invention necessitated numerous examples was erroneous. *Id.* Of particular relevance to the issues at hand, the court held that although the invention is applicable to a large variety of haptens and antigens, the examiner offered no reason why these different compounds would require different techniques or process parameters. *Id.* As explained in detail below, the evidence of record has demonstrated the fact of the phenomena underlying the invention.

The Examiner's allegations of non-enablement are based upon un-scientific evidence such as a business newsletter, publications which provide explanations and/or results that are actually contrary to the Examiner's position, and publications that are not related to the methods at issue in this case.

By contrast, Applicants have provided reasoned refutation of the Examiner's contentions which can be found in the references cited by the Examiner and experimental evidence of enablement provided in declarations of the inventor.

1. Claims 52, 59-61, 63, 69-91, 95, And 102 Are Patentable Under The Enablement Requirement Of 35 U.S.C. § 112, First Paragraph

The Examiner has alleged that the disclosure of the instant specification is insufficient to enable one skilled in the art to practice the invention as claimed without undue experimentation. *See* OFFICE ACTION DATED APRIL 14, 2009, at 2, the final paragraph. Applicants disagree with the Examiner for at least the reasons set out below.

The Examiner has asserted that the claims of the present application are intended solely for use in humans with respect to transplantation antigens as only humans undergo transplantation and based conclusions of non-enablement on this proposition. *See* OFFICE ACTION DATED APRIL 14, 2009, at 3. Applicants respectfully disagree with the Examiner's interpretation of the claims, since the antigens of the presently claimed invention include both transplantation antigens and autoantigens. Moreover, transplantation may be conducted in any mammal as desired and is not simply limited to humans. Transplantation in veterinary medicine is becoming more commonplace. Indeed, the technology has been licensed to Dow AgroSciences for applications in companion animals. *See* Exhibit 1. Thus, there is no basis for the Examiner's claim construction.

Moreover, the Examiner has not demonstrated good reason to believe that use of the claimed invention to improve tolerance in transplant patients would require undue experimentation.

The Examiner has further alleged that, as of 1994, there were no methods available for inducing oral tolerance to a transplantation antigen in a human. *See* OFFICE ACTION DATED APRIL 14, 2009, at 3. The statement is true as regards the claimed invention, as the present application discloses for the first time a method for suppressing or reducing the immune response of a mammal by administering a plant tissue or plant tissue extract as claimed. However, the Examiner's contention is contrary to the prior determination of the

Office. The United States Patent and Trademark Office has previously determined that a method for suppressing the immune response of a recipient mammal to non-self tissue from a donor mammal comprising: orally or enterally administering to said recipient mammal an agent in an amount effective for suppressing said immune response, said agent comprising a MHC antigen or a peptide fragment of a MHC antigen said MHC antigen being from the donor of said non-self tissue or syngeneic to the donor of said nonself tissue was enabled based on a disclosure filed in 1990. *See* U.S. Patent No. 5,788,968 (Exhibit 22), corresponding to WO 92/07581 (“Weiner et al.” attached hereto as Exhibit 21).

The Examiner has also alleged that, some 15 years later, “therapeutic tolerance has not been reproducibly demonstrated to be inducible in humans”. The Examiner’s contention is contradicted by his own acknowledgment of at least two examples that demonstrate therapeutic tolerance. Specifically, the Examiner acknowledges “the possible exception of some allergy and Rh antigens” (emphasis added). *See* OFFICE ACTION DATED APRIL 14, 2009, at 3, first paragraph. Thus, by his own admission, there exist examples demonstrating that therapeutic applications of the claimed methods do exist. As was the case in the *Strahilevitz* case, the Examiner has failed to provide evidence that any other antigens require different treatment or undue experimentation, and thus the rejection must fail. *See In re Strahilevitz*, 212 USPQ 561 (C.C.P.A. 1982).

However, even if the Examiner might be considered to have made out a prima facie case. Applicants have provided evidence of experiments conducted in accordance with the teachings of the specification which demonstrate that transgenic plants expressing autoantigens induced oral immune tolerance when fed to mice. Evidence of these experiments was presented in declarations submitted by Dr. Jevnikar, an inventor of the claimed invention, in the parent application and again in the present application in the response dated July 12, 2004. A further declaration was submitted by Dr. Jevnikar in the

response dated February 8, 2005. Exhibits 2, 3 and 4 attached hereto, hereafter referred to as the “1999 Jevnikar Declaration”, the “2000 Jevnikar Declaration”, and the “2005 Jevnikar Declaration”, respectively.

The 1999 Jevnikar Declaration, states that the inventors of the present application “have found that plant material obtained from a transgenic plant expressing a mammalian autoantigen can be used to produce oral tolerance”. Exhibit 2, at ¶¶ 13-16. This finding of the present inventors was further described in Ma et al., *Nature Medicine*, 1997, v. 3, p. 793-796 (Exhibit 5 attached hereto). In the 1999 Jevnikar Declaration, further data was provided, indicating that “plant material containing plant-expressed transgenic GAD gave enhanced T cell activation, which is a pre-requisite step in the induction of immune tolerance, including oral tolerance”. It is also detailed by Dr. Jevnikar that “oral administration of plant material obtained from a transgenic plant expressing a mammalian transplantation antigen or autoantigen results in administration of not only of the recombinant antigen but also of additional plant components which may assist in the induction of oral immune tolerance. For example, plant lectins are bound to nucleated cells of the gut and lectins are known to arrive intact in the small intestine, where there are lymphoid cells involved in the induction of oral tolerance”.

The 2000 Jevnikar Declaration provided further clarifying comments and even more data supporting the enablement of the presently claimed invention. This data shows that “there is an unexpected synergistic enhancement of T cell activation when GAD-reactive T cells are primed with plant material containing expressed GAD”. Exhibit 3, at ¶ 15.

As was detailed in the 2005 Jevnikar Declaration, the animal data which has been provided in the present application would be understood by those of skilled in the art to support the use of the claimed methods and compositions in humans. Exhibit 4, at ¶ 7. Additionally, the experiments described in the 1999 and 2000 Jevnikar Declarations, which

demonstrate favourable and beneficial results in mice, involve a credible *in vivo* model that correlates with humans and is often used by those of skill in the art. Exhibit 4, at ¶ 6.

The Examiner appears to have disregarded the evidence presented in the Jevnikar Declarations and has argued that “a review of the instant specification shows no induction of tolerance” as the present application does not provide any *in vivo* or *in vitro* data. See OFFICE ACTION DATED APRIL 14, 2009, at 5-6. Applicants submit that it is well recognized by the Federal Circuit that *in vivo* testing is not necessary to satisfy the requirements of § 112, first paragraph, as this confuses the requirements under the law for obtaining a patent with the requirements for obtaining government (FDA) approval to market a particular drug for human consumption. *In re Brana*, 34 USPQ2d 1436 (Fed. Cir. 1995). The evidence described above presented in the declarations of Dr. Jevnikar regarding experiments that have been conducted clearly demonstrating that transgenic plants expressing autoantigens induced an oral immune response when fed to mice, as is presently claimed, cannot be ignored.

Having ignored the evidence presented by applicants, the Examiner has alleged that the presently claimed invention is not enabled because “attempts to induce tolerance in humans have been completely unsuccessful in multiple different documented instances”. See OFFICE ACTION DATED APRIL 14, 2009, at 3. The Examiner has cited several references in the Office Action, each of which is discussed in detail below.

2. The References Cited in the Record of the Application Do Not Support the Rejection

Notably, none of the references referred to by the Examiner administer antigen in the form of transgenic plant tissue or transgenic plant tissue extracts, as is presently claimed. As discussed above, this means that none of the cited references used the synergistic combination of plant material and antigen to induce oral tolerance in animals or humans, as is presently claimed. For at least this reason, none of these references can be taken as evidence

that the presently claimed invention is unpredictable or not enabled. Moreover, reading of the references beyond the passages cited by the Examiner demonstrates that the Examiner appears to have improperly picked and chosen specific facts and quotes from these references that do not represent their overall teachings. Indeed, a detailed and careful reading of these references supports, rather than refutes, the enablement of the presently claimed invention.

(a) Marketletter, 1999

The Marketletter reference (attached hereto as Exhibit 6) is a business newsletter, not a scientific publication. As this is not a scientific publication and is thus not peer-reviewed, it cannot be considered to be a reliable scientific document. A person of ordinary skill in the art would not consider such a reference, which discusses business markets in order to help people decide where to invest their money, as a scientific document that can refute the enablement of a claimed invention.

Furthermore, this newsletter read as a whole does not support a conclusion that attempts to induce oral tolerance in humans were a “complete failure” or were “completely unsuccessful” as the Examiner alleges. *See* OFFICE ACTION DATED APRIL 14, 2009, at 3. Instead, the newsletter states that “Colloral was found to be safe” and “that ‘substantial improvements’ from baseline were observed” in each of the measured endpoints. MARKETLETTER, at ¶ 3. Unfortunately, a high response in the placebo group masked this positive outcome and statistical significance was not reached. Despite the lack of statistical significance, Colloral must have had some positive outcomes as the owners were still “considering switching the focus of Colloral to a nutraceutical product”. *See* MARKETLETTER 6, at ¶ 3. Indeed, the owner stated that “it still firmly believes in its technology” and that “both basic and clinical research focused on enhancing the biological effect of [mucosal tolerance therapy] in patients will continue”. *See* MARKETLETTER, at ¶ 10.

Contrary to the allegations of the Examiner, this reference does not describe the complete failure of an attempt to induce oral tolerance in humans but, rather, a marginal success that was not immediately further pursued due to financial concerns. In contrast to the Examiner's allegation, this reference actually supports Applicant's position that the presently claimed invention is enabled.

(b) Pozzilli et al., 2000

The Examiner has cited Pozzilli et al. (attached hereto as Exhibit 7) alleging that the trial described in this reference was a failure and that "both the treated and control subjects showed similar temporary improvement." Applicants again disagree with the Examiner. The results of the trial described by Pozzilli et al. did not achieve statistical significance. However, Pozzilli et al. gave explanations for why statistical significance may not have been achieved that do not implicate failure of the treatment. For example, the dose of oral insulin used may have been too low, no adjuvant had been used, and the level of beta cell mass may have already been too low at the time the trial began for the treatment to have any meaningful effects. See Pozzilli et al., page 1003, col. 2 to page 1004, col. 1. In any event, a lack of statistical significance does not mean that the trial of Pozzilli et al. was a "failure" or that the presently claimed invention is not enabled. In fact, a critical review of the data indicates a trend towards the induction of tolerance in humans by oral administration of antigen.

In particular, Figure 2 shows the level of insulin antibodies found in patients treated with oral insulin (grey bars) versus those treated with placebo (white bars). At each timepoint tested, i.e., 3, 6, and 12 months, the mean amount of insulin antibodies was lower in patients treated with oral insulin than in those treated with placebo. At the 12 month timepoint, patients treated with placebo had approximately 240 units of insulin antibodies, whereas patients treated with oral insulin had approximately 80 units of insulin antibodies. This is a reduction in mean insulin levels of 67% following treatment with orally

administered antigen. Although there was a great deal of variability amongst the patients, making statistical conclusions difficult, this mean reduction is evidence of a trend towards the induction of oral tolerance. Accordingly, this reference, like the Marketletter reference, supports Applicant's position that the presently claimed invention is enabled.

(c) Skylar et al., 2005

The Examiner states that Skylar et al. (attached hereto as Exhibit 8) "reported another failure in one of the largest placebo-controlled tolerance trials ever performed in humans (the administration of insulin for the prevention of type I diabetes)". However, Applicants submit that what the Examiner concludes is a failure is not the conclusion that was reached by the authors of the reference. In fact, Skylar et al. state that, although the results were not statistically significant, one specific group of human patients "showed a beneficial effect of oral insulin". See page 1073, col. 3, paragraph 2 of Skylar et al. This data was so promising that the authors state that the successor study group "is contemplating a confirmatory study to explore the potential role of oral insulin in delaying or preventing type I diabetes in relatives found to be at risk for diabetes with IAA levels similar to those in the DPT-1 subgroup". See Skylar et al. at, 1073-74. Contrary to the Examiner's contention, this study was not a "failure." The authors conclude that "inclusion of subjects with variable and lower risk of diabetes...may have masked a treatment effect", making it difficult to reach statistical significance. However, as was noted above, a beneficial effect of orally administered antigen was observed in at least one specific cohort of patients. Accordingly, this reference supports Applicant's position that the presently claimed invention is enabled.

(d) Dong et al., 1999

Dong et al. (attached hereto as Exhibit 9) is a review article that discusses transplantation tolerance in general terms. The induction of oral tolerance is listed amongst many "Novel Clinical Strategies" for achieving transplantation tolerance. Specifically, at

page 186, col. 1, paragraph 1, Dong et al. state that “*In vivo* studies have demonstrated reduced delayed-type hypersensitivity responses to a mixture of polymorphic class II MHC allopeptides in peptide immunized rats with oral administration of the peptide mixture prior to immunization.... Whether tolerizing to MHC peptides, by oral administration for example, will have an impact on delaying progression of chronic rejection remains to be seen”. As noted by the Examiner, Dong et al. speculate that, in transplant patients, it is difficult to assay for true tolerance because withdrawal of immunosuppressive agents would compromise patients in which tolerance has not occurred. However, this does not mean that the presently claimed invention lacks enablement. Rather, this simply describes an ethical concern when one is faced with testing a novel therapy that would require stopping a clinically accepted therapy. Dong et al. provide no evidence that the presently claimed invention is not enabled.

(e) WO 02/053092

WO 02/053092 (attached hereto as Exhibit 10) discloses a method of inducing immune tolerance to plaque associated molecules such as LDL, beta-2-GPI, and HSP in humans. The Examiner refers to page 23 of the ‘092 application, asserting that the inventors concluded that “oral and mucosal tolerance cannot be deduced from antigenic activity in conventional immunization, or even *in vitro* results, and must result from extensive empirical experimentation”. Applicants submit that the Examiner has taken this quote out of context and has considered it in a vacuum. In actual fact, this quote was made with respect to a summarized description of the prior art and has nothing to do with the enablement of the presently claimed invention. Moreover, the ‘092 application states that “oral and mucosal tolerance for the suppression and prevention of inflammatory conditions is well known in the art”. WO 02/053092 at, 22, lines 25-27. Ultimately, this application shows that immune

tolerance can be accomplished. Accordingly, this reference supports Applicant's position that the presently claimed invention is enabled.

(f) Goodnow, 2001

Goodnow, 2001 (attached hereto as Exhibit 11) is a review article that provides an overview of self-tolerance pathways and is not a description of a specific experiment that is designed to induce tolerance. The Examiner has quoted a sentence from the abstract of this reference, which states that "Obtaining the desired response with these strategies is unpredictable because many of these signals have both tolerogenic and immunogenic roles". The Examiner has unilaterally inferred that "the desired response" is "tolerance", that "these strategies" are "tolerance induction", and that "these signals" are "tolerogenic signals". Respectfully, this is incorrect. When taken in the context of the remainder of the abstract, it is clear that the unpredictability described by Goodnow refers to the use and mechanism of action of corticosteroids and other new experimental therapies. The induction of oral tolerance, as is presently claimed, is not mentioned in the abstract. Moreover, the use of plant tissue and/or extracts for administration of antigen, as is presently claimed, is not mentioned once in this entire reference.

The Examiner has specifically referred to page 2120, col. 2, paragraph 3 of Goodnow, stating that Goodnow teaches that "while the induction of oral tolerance might be considered 'an attractive notion', the method has failed in humans because of the lack of understanding of the mechanisms involved". *See* OFFICE ACTION DATED APRIL 14, 2009, at 4. However, Goodnow's teachings are much more positive than the Examiner's summary would imply. Goodnow, in fact, states that "In animal models, delivering low amounts of antigen by the mucosal route, either ingested or nasally, can act as a potent tolerogen". Goodnow concedes that "The first clinical trial of oral tolerance was unsuccessful", however, Goodnow does not indicate that he would expect all clinical trials to fail nor does Goodnow indicate that the

presently claimed invention, using plant tissues and/or extracts for oral or enteral administration of antigen, would fail. Instead, he states that there is a “need to understand better the mechanisms involved and to develop ways to achieve more reliable linkage between tolerogenic antigen and suitable tolerogenic stimuli”. Goodnow also teaches, at page 2118, col. 1, paragraph 2, that “Clinical trials are underway aimed at preventing type 1 diabetes or ameliorating multiple sclerosis by inducing oral tolerance to pro-insulin or myelin basic protein”. Clearly, Goodnow supports the general proposition that the induction of oral tolerance is achievable. Perhaps more importantly, Goodnow neither provides nor cites any empirical data that convincingly refutes that the induction of oral tolerance by administration of antigen orally or enterally by ingestion of plant material, as is presently claimed, is achievable.

(g) Kraus and Mayer, 2005

Kraus and Mayer, 2005 (attached hereto as Exhibit 12) is a review article discussing oral tolerance induction in patients with Crohn’s disease, ulcerative colitis, or inflammatory bowel disease (IBD). The Examiner states that this reference teaches that there is “a genetic component wherein many IBD patients and their family members appear to be incapable of becoming tolerant to oral antigens because they lack the ability to generate the required T regulatory cells”. The Examiner then speculates that, “If confirmed, this would mean that no tolerance induction regime could work in these patients”. *See* OFFICE ACTION DATED APRIL 14, 2009, at 5. However, the Examiner’s speculation is completely contrary to the whole of the teachings of Kraus and Mayer. Indeed, at page 694, col. 1, paragraph 2, Kraus and Mayer stated that “all the normal control subjects tolerized” although several of the Crohn’s disease and ulcerative colitis subjects did not. However, even though many of these subjects did not tolerize, some certainly did. This clearly indicates that it is possible to induce oral tolerance

in this cohort of subjects. Moreover, the standard of enablement does not require 100 % efficacy for 100 % of subjects. Such results are rarely if ever achieved

Kraus and Mayer also report that further studies have been even more successful. In the paragraph bridging cols. 1 and 2 of page 694, Kraus and Mayer state that “after a 16-week treatment of three feedings a week, a median decrease in the CDAI of 129 [from initial scores of between 200 and 350] after 14 weeks was observed”. These are positive results indicating that the induction of oral tolerance can be achieved in patients with Crohn’s disease. In fact, Kraus and Mayer report that these results were so positive that “further multicenter trials are pending from this company”.

Kraus and Mayer discuss the differences between the induction of oral tolerance in animal models and in human subjects and state that “although the phenomenon of oral tolerance might be shared between species, the mechanisms of oral tolerance seem to differ”. *See Exhibit 12, at 694, col. 1, ¶ 1.* This statement was made in reference to differences in B cell responses in mice and humans. Kraus and Mayer teach that oral tolerance can be achieved in both humans and mice and that certain B cell responses may differ between these species. Additionally, Kraus and Mayer state that the reasons why oral tolerance therapy has not been as successful in humans as it has been in mice are unclear. However, Kraus and Mayer speculate that “it is possible that the oral tolerance response in humans, using the doses and feeding schedule use in the trials, is not optimal. The optimal oral tolerance response in humans has not yet been fully explored”. This reference does not mention administering antigen together with plant material, as is presently claimed. As discussed above and in the 1999 and 2000 Jevnikar Declarations, this co-administration results in a synergistic effect with respect to tolerance. Thus, this reference does not support the Examiner’s argument that the induction of oral tolerance in humans cannot be achieved.

Rather, as discussed above this reference supports the contention that oral tolerance can be induced in humans.

(h) Bell et al., 2008

Bell et al. 2008 (attached hereto as Exhibit 13) is directed to the induction of tolerance in mice by administration of antigen intraperitoneally, i.e., parenterally. See Exhibit 13, at 1509, col. 2, paragraph 1: “the treatments were given on days 13, 17, 21 postdisease induction by i.p. injection”. The teachings of this reference are thus completely irrelevant to the presently claimed invention, wherein antigen is administered orally or enterally. Thus, this reference does not support the Examiner’s contention that the presently claimed invention is inherently inoperative.

(i) Husby et al., 1994

Husby et al. 1994 (attached hereto as Exhibit 14) reports a successful trial of oral tolerance induction in human patients. The Examiner acknowledges that “the reference teaches the reduction of *in vitro* T cell proliferation and delayed skin test responses to KLH”. See OFFICE ACTION DATED APRIL 14, 2009, at 6. However, the Examiner argues that “no reduction in B cell responses was observed”. In view of this, the Examiner alleges that “the reference cannot enable the broad methods and compositions of the instant claims that recite the suppressing or reducing of any type of immune response”. Applicants disagree with the Examiner’s conclusion. As noted above, “suppressing or reducing the immune response of a mammal to an antigen”, as is presently claimed, means that the mammal’s tolerance to that antigen is increased.

Contrary to the Examiner’s implication, the study of Husby et al. demonstrates induction of tolerance. In as much as there were delayed skin test responses by the patients to KLH, the antigen used in Husby et al.’s study, clearly tolerance to that antigen was increased. How that result is achieved, i.e., through T cell or B cell responses, is completely irrelevant.

The fact is that this reference teaches that oral tolerance can be achieved. Indeed, as was stated in Husby et al., at page 4667, col. 1, paragraph 1, “The present study demonstrates that oral tolerance can be induced in humans”.

The conclusion of Husby et al., a group of scientists skilled in the art is in fact contrary to the assertions of the Examiner. In fact, this reference supports the position that the presently claimed invention is fully enabled.

(j) Moldoveanu et al., 2004

Moldeveanu et al. 2004 (attached hereto as Exhibit 15) is a study of antigen feeding in a small cohort of patients that had a pre-existing immune system condition. The Examiner has pointed to the final sentence of the abstract, which states that “some form of immunomodulation greater than that provided by oral administration of antigen alone is required in humans for suppression of an existing immune response”. *See* OFFICE ACTION DATED APRIL 14, 2009, at 7.

However, the presently claimed invention is not directed to the administration of antigen alone, as was done in Moldoveanu et al. Instead, the presently claimed invention is directed to the administration of antigen together with plant tissue or plant tissue extracts, which has been shown to provide a synergistic inducement of oral tolerance. See the 1999 and 2000 Jevnikar Declarations. Thus, Moldoveanu et al.’s single reporting of an unsuccessful trial that administered antigen in a completely different manner from that which is presently claimed cannot be said to support the Examiner’s position that the presently claimed invention is not enabled.

In fact, contrary to the Examiner’s position, Moldoveanu et al. concluded that “the effective use of oral tolerance to treat human disease will require adjuvants and delivery systems, just as is required for oral immunization”. See Exhibit 15, at 308, paragraph 3. Such adjuvant delivery systems are inherent in the presently claimed invention, which is

administered in the form of plant tissues or plant tissue extracts. Thus, Moldoveanu et al. again confirms that the presently claimed invention is enabled.

(k) McKown et al., 2000

McKown et al. 2000 (attached as Exhibit 16) teaches the induction of immune tolerance in humans by oral administration of bovine type I collagen. The Examiner has acknowledged that “the reference provides encouraging preliminary data indicating the oral administration of type I collage (CI) might be useful for treating systemic sclerosis (SSc)”. *See* OFFICE ACTION DATED APRIL 14, 2009, at 7. The Examiner then somehow dismisses the findings of this reference as “having not risen past the level of idea”. *Id.* Applicants respectfully disagree. McKown et al. teach that “[t]aken together, these data indicate that oral tolerance to CI was effected by this treatment regimen”. *See* Exhibit 16, at 1059, col. 2, ¶ 1. In this same paragraph, the authors described the reduction of serum levels of sIL-2R “after induction of oral tolerance to CI”, which further confirms the conclusion that the authors themselves believe that the treatment induced oral tolerance. Thus, a reading of the whole of this reference shows that oral tolerance was achieved.

The Examiner further points to the reduction in IL-10 identified in this study. The Examiner refers to this reduction as “unexplainable”. Applicants respectfully submit that the Examiner is incorrect. The authors provide various explanations as to why IL-10 may have been reduced in this study. *See* Exhibit 16, 1059, col. 2, ¶ 1. The Examiner has also pointed to broad statements in the final paragraph of the reference that refer to future trials. The fact that additional study is desirable is in the nature of scientific endeavour. The desire to improve the methods does not refute the teachings of the remainder of this reference in which the authors concluded that oral tolerance was achieved. Again, McKown et al. 2000 actually supports a finding of enablement of the presently claimed invention.

(I) McKown et al., 1999

McKown et al. 1999 (attached as Exhibit 17) reports a study wherein patients with rheumatoid arthritis (RA) were fed bovine type II collagen (CII). These patients did not exhibit oral tolerance following treatment. The authors concluded that this was likely because the patients were being concomitantly treated with a range of drugs, including disease-modifying antirheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and prednisone, which the authors recognized as compounds that suppress immune response.

Thus, contrary to the Examiner's implication that the study shows a failure, authors themselves noted that previous "studies by Trentham et al. and Barnett et al. showed significant improvement in arthritis" and that "the major difference between the design of the present study and the 3 earlier trials is that DMARDs, which were taken by 85% of the patients in our study, were not discontinued". See Exhibit 17, at 1207, col. 1, last paragraph, to col. 2, first full paragraph.

The authors also reasoned that the dose used in their study may have been too low and that "NSAIDs and prednisone used to treat RA, and which have been allowed to be continued during each of the CII trials reported to date, may interfere with the induction of oral tolerance." See Exhibit 17, at ¶¶ bridging pp. 1207-08. Noting further that "[i]t has been previously shown in different animal models that NSAIDs abrogate peripheral and oral tolerance induction." *Id.* Thus, the explanations of the authors are consistent with an understanding in the art that oral tolerance is a phenomenon that is to be expected and that its absence is an anomaly to be explained.

Moreover, as has been noted above in respect to other references cited by the Examiner, the antigen used by McKown et al. was not administered in the manner that is presently claimed, using plant tissue or plant tissue extract. Instead, the antigen was

administered alone. Thus, even if the Examiner's contentions with regard to McKown et al. 1999 were true, such a result would not be evidence that the presently claimed invention is not enabled in view of the teachings of this reference.

(m) Carbone et al., 2004

Carbone et al., 2004 (attached hereto as Exhibit 18) is a letter to the editor of the journal "Arthritis and Rheumatism", reporting on a study using lower doses than those used in the McKown et al., 2000 reference discussed above. Notably, in the first paragraph, the authors refer to the success of this previous study in inducing oral tolerance to type I collagen (CI) in humans. In the second paragraph, Carbone et al. state "in our previous study of oral CI administration to patients SSc, we used high doses of collagen (500 µg/day) and did not explore the effects of lower-dose regimens. The purpose of the present study was to determine whether lower doses of oral CI (10 µg/day and 100 µg/day) would induce immune tolerance to CI in patients with SSc". Perhaps not surprisingly, the authors found that such low doses of CI used over a relatively short period of only 5 months, had little effect on the patients' immune systems. However, Carbone et al. state that "with the 100 µg/day dosage of CI, we did note increased in vitro production of IFNγ.... Increased IFNγ production by peripheral lymphoid cells cultured with tolerizing antigen has been associated with induction of tolerance to some orally administered antigens in humans and mice". See page 2714, col. 2, last paragraph of Carbone et al. Therefore, the Examiner's comment on page 7 of the Office Action that "the oral administration of CI had no effect on SSc patients" is completely unfounded.

Moreover, the Examiner's allegation that the authors were "simply employing methods of trial-and-error (unsuccessfully)" is incorrect. The authors were systematically testing various dosages and found that the high dose of 500 µg/day was the most successful, followed by the dose of 100 µg/day. It is predictable, and indeed taught in the present

application, that higher doses of antigen would be expected to yield better results than low doses of the same antigen. *See* SPECIFICATION at 2, lines 21-26. Experimentation to determine appropriate dosing is neither undue nor is it an indicator of the alleged “unpredictability” of the claimed methods. Rather, it is a reasoned approach to determine the efficacy of lower concentrations of collagen I and, in so doing, to improve the technology. Experiments seeking to improve a technology cannot be held to show that the technology is not enabled.

(n) Womer et al., 2008

Womer et al., 2008 (attached hereto as Exhibit 19) reported

We demonstrate complete suppression of baseline indirect alloreactivity in patients with chronic renal allograft dysfunction following the oral feeding of...donor major histocompatibility complex (MHC) class II peptides.... Our results indicate that oral feeding of low dose donor MHC peptide may represent a safe and effective therapy to suppress indirect alloreactivity in renal transplant recipients with chronic allograft dysfunction and warrants further clinical investigation.

See the abstract. The Examiner has completely discounted the findings of this reference alleging that there were too few participants, the study did not last long enough, and that the results were transient. Thus, the Examiner appears to admit that, even though the study was short and only involved a few patients, transient oral tolerance was in fact achieved. This would support the enablement of the presently claimed invention, suggesting that a positive result was obtainable even after a short period of time and with a small sample size. This reference supports the Applicant’s position that the presently claimed invention is enabled.

(o) Ergun-Longmire et al., 2004

Ergun-Longmire et al., 2004 (attached hereto as Exhibit 20) reports a study of the effects of oral insulin on the progression of type I diabetes. At page 9 of the Office Action, the Examiner quotes this reference, stating that “Disappointingly, there were no clinical benefits discernible from our oral insulin tolerance therapy as reflected in improved diabetes

control, lowered glycated haemoglobin levels, or reduced daily insulin dosage”. The Examiner then concludes that this is an indication that “the method did not work”.

Applicants respectfully submit that the Examiner is in error. A full reading of this reference indicates that “among subjects 20 years of age and older, there was a statistically significant benefit of treatment with 1 mg oral insulin ($P=.002$), even after adjusting for baseline C-peptide values ($P=.003$; Fig. 3B). There was a smaller, yet statistically significant benefit ($P=.01$) from treatment with 10 mg oral insulin as well in this group”. See page 273, paragraph 2). The authors conclude that “our study indicates that retention of endogenous insulin secretion after diagnosis of type 1 diabetes presenting after the pubertal years can be induced by the sustained, daily ingestion of oral insulin”. See Exhibit 20, at 274, ¶ 1. In summary, the authors state that “ours is the first report to suggest a benefit from oral insulin tolerance therapy in newly diagnosed type 1 diabetic patients”. See Exhibit 20, at 275, final ¶. The Examiner’s conclusion that “the method did not work” is unsupported by the actual results, which demonstrated induction of oral tolerance. The teachings of this reference, in fact, support the Applicant’s position that the presently claimed invention is fully enabled.

3. The Examiner’s Unsupported Contentions are in Error

The Examiner has further alleged as follows:

And finally consider the simple fact that if all that was required to establish immune tolerance was the oral or enteral administration of antigen, then there would be no food allergies and no allergies to airborne allergens like dust mites and ragweed – everyone would be tolerant because of repeated environmental administration.

See OFFICE ACTION DATED APRIL 14, 2009, at 6, ¶ 2. This bald conclusory opinion is presented without any supporting scientific reference.

The Examiner’s contention is contrary to scientific fact demonstrated throughout the references discussed above and in the declarations of Dr. Jevinikar submitted in this application. Indeed, all that is required to establish immune tolerance is the oral or enteral

administration of antigen. This is why most people are not allergic to the vast majority of the things that they eat and inhale each and every day.

For example, consider Husby et al., 1994, discussed above, which clearly teaches that “oral tolerance may represent an important immunoregulatory process that limits immune response to innocuous food Ags [antigens]. Certainly, humans ingest food Ags daily in quantities that should result in tolerance, and a small fraction is known to be absorbed into the circulation”. See Husby et al. at 4668, col. 2, last ¶.

Consider also McKown et al., 1999, also discussed above, in which the authors stated that “The GALT [gut-associated lymphoid tissue] is particularly adapted to preventing harmful immune responses to the myriad of dietary antigens ingested during a normal lifespan. This hyporesponsiveness to ingested antigens is produced by a phenomenon known as oral tolerance”. See McKown et al., 1999, at 1204, col. 2, ¶ 1.

The weight of the evidence is contrary to the Examiner’s unsupported contentions. The present application teaches and the Jevnikar Declarations provide evidence that oral tolerance can be synergistically induced by administering such antigens together with transgenic plant material. Therefore, the Examiner’s unsupported contention should be given no weight. “The Examiner should **never** make the determination based on personal opinion.” M.P.E.P. § 2164.05 (emphasis in original). The determination should always be based on the weight of all the evidence. *Id.*

4. The Enablement Rejection Should Be Reversed

In summary, the fact of the phenomena that underlies and enables the claimed methods and products is never denied in any publication cited by the Examiner. At most, the cited documents demonstrate the results of experimentation in the effectiveness of particular varying treatment parameters. Even if experimentation to optimize treatment parameters may be complex, it does not necessarily make it undue, if the art typically engages in such

experimentation. *See, e.g., In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983) *aff'd sub nom., Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Circ. 1985). *See also, In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. The test of enablement is not whether any experimentation is necessary but, whether, if experimentation is necessary, it is undue. *See In re Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219 (CCPA 1976); M.P.E.P. § 2164.01. Moreover, the enablement requirement does not require that every variation of a method produce optimal results.

Applicants have provided more than sufficient documentary and testimonial evidence to refute the allegations of the Examiner. Evidence provided by the Applicant need not be conclusive but merely convincing to one skilled in the art. M.P.E.P. § 2164.05. A declaration or affidavit is, itself, evidence that must be considered. *Id.* Dr. Jevnikar's submitted declarations are supported by evidence published in peer reviewed journals that oral tolerance has been achieved in humans. *See, e.g.,* Husby et al., 1994 and McKown et al., 2000, discussed in detail above. By contrast, none of the references cited by the Examiner administer antigen together with transgenic plant tissue, as is presently claimed. Therefore none of the Examiner's purported evidence may be taken as conclusive with respect to the present invention.

Applicants have provided references demonstrating that oral tolerance can be achieved in mammals. Applicants have demonstrated the expression of antigen in plants. Applicants have provided evidence of enablement in an art accepted animal model. Applicants have demonstrated that plant material containing expressed GAD, administered orally, suppresses or reduces the immune response of a mammal to the autoantigen GAD. This evidence should not be discounted by the Examiner. Even if, as the Examiner asserts, there have been reports of less than optimal results under some experimental protocols, it

cannot be reasonably disputed that oral tolerance has been achieved. The weight of the evidence as a whole leads to the conclusion that the pending claims have been demonstrated to be enabled.

In view of at least the above and all the evidence submitted, including the three Jevnikar Declarations, the present application provides sufficient enablement so that one skilled in the art could make and/or use the claimed invention without undue experimentation. Thus, the rejection under 35 U.S.C. § 112, first paragraph, should be reversed and such action is respectfully requested.

B. Rejection Under 35 U.S.C. § 112, First Paragraph, Written Description

Claims 52, 59-61, 63, 69-76, 78-91, 95, and 102 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in relevant art that the inventor, at the time the application was filed, had possession of the claimed invention.” *See* OFFICE ACTION DATED APRIL 14, 2009, at 13.

1. Claims 52, 59-61, 63, 69-76, 78-91, 95, And 102 Are Patentable Under The Written Description Requirement Of 35 U.S.C. § 112, First Paragraph

The Examiner has alleged that “there is insufficient written description to show that Applicant was in possession of an “immunosuppressive fragment” of an antigen as is set forth in the claims”. *See* OFFICE ACTION DATED APRIL 14, 2009, at 13. Applicants disagree with the Examiner for at least the reasons set out below.

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. *See, e.g., Moba, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306, 1319, 66 USPQ2d 1429, 1438 (Fed. Cir. 2003). An

“immunosuppressive fragment” as recited in the claims, is explicitly defined in the Specification. *See* SPECIFICATION, at 7, lines 30-35.

There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed. *In re Wertheim*, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976) (“we are of the opinion that the PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims”). In this case, there is explicit disclosure in the specification of the element which the Examiner has alleged is not described.

An “immunosuppressive fragment” is defined in the specification as “a portion of the amino acid sequence of the protein or polypeptide which is capable, on oral or enteral administration to a mammal, of inducing tolerance or suppressing the immune response of the mammal to the protein or polypeptide.” *See* SPECIFICATION, at 7, lines 30-35. The specification describes a representative variety of antigen proteins or polypeptide, for example, at page 9, lines 5-8, page 10, lines 3-9, page 10, lines 15-25, page 10, line 34 to page 11, line 11. The structures and sequences of such antigens would be known to those of skill in the art. A person of ordinary skill would understand that an immunosuppressive fragment is merely a fragment of such an antigen that contains a sufficient portion of the antigen to promote tolerance in accordance with the invention.

Applicants submit that a person of ordinary skill in the art would readily understand from the disclosure that whole proteins or polypeptide antigens can be broken into fragments and that a fragment retaining a sufficient portion of the polypeptide to provide an immune suppression response by the methods of the present invention is an immune suppressive fragment. Persons of ordinary skill would understand from common sense that when a protein is fragmented below a reasonable size, there would be less expectation that the

fragment would produce the same immune suppressive effect as the whole antigen. Thus, a person of ordinary skill in the art would have no reason to doubt that the inventors were in possession of the claimed invention, including the use of fragments of the various antigens described in the specification, at the time the application was filed.

2. The Reference Relied Upon By the Examiner Does Not Support the Rejection

In support of this rejection, the Examiner has referred to Bell et al. (Exhibit 13), discussed above, and to the enablement rejection raised in the Office Action. The Examiner contends that Bell et al. shows that whether a fragment of an antigen is immune suppressive is unpredictable. However, the results of Bell et al. cannot be used to draw any conclusions regarding the present invention. As noted above, Bell et al. is the results of Bell at al. are irrelevant to the presently claimed invention because antigen was administered by injection in Bell et al. Therefore, the antigen of Bell et al. does not encounter the intestinal epithelium as it would in the presently claimed methods. As such, this reference cannot be relied upon as an indication of unpredictability in the methods and compositions of the present invention.

3. The Written Description Rejection Should Be Reversed

In view of at least the above the present application provides sufficient written description to show that Applicant was in possession of an “immunosuppressive fragment” of an antigen as is set forth in the claims. The Examiner’s purported evidence is not effective. Thus, withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, is respectfully requested.

C. Claim Rejections Under 35 U.S.C. §103 - Obviousness

Claims 52, 59-61, 63, 69-76, 78-91, 95, and 102 stand rejected under 35 U.S.C. §103(a) as being unpatentable over WO 92/07581 (“Weiner et al.” attached hereto as Exhibit

21) in view of U.S. 5,484,719 (“Lam et al.” attached hereto as Exhibit 22). *See* OFFICE ACTION DATED APRIL 14, 2009, at 9.

The basic factual inquiries that must be made to determine obviousness or non-obviousness of patent application claims under 35 U.S.C. §103. are set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 17, (1966):

Under § 103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; the level of ordinary skill in the pertinent art resolved. Against this backdrop, the obviousness or non-obviousness of the subject matter is determined.

Appellants respectfully submit that the factual inquiries set forth in *Graham* have not been considered or properly applied by the Examiner formulating the rejection of the pending claims. Particularly the differences between the claims and the prior art as a whole were not properly determined. *See, e.g. In re Ochiai*, 37 U.S.P.Q.2d 1127, 1131 (Fed. Cir. 1995)(“[t]he test of obviousness *vel non* is statutory. It requires that one compare the claim’s subject matter as a whole with a prior art to which the subject matter pertains.”).

When rejecting claims under 35 U.S.C. §103, an Examiner bears an initial burden of presenting a *prima facie* case of obviousness. If an Examiner fails to establish a *prima facie* case, the rejection is improper and will be overturned. *See: In re Rijckaert*, 9 F.3d 1531, 1532, 28 U.S.P.Q.2d. 1955 (Fed. Cir. 1993). “If examination.... does not produce a *prima facie* case of unpatentability, then without more the applicant is entitled to the grant of the patent.” *In re Oetiker*, 977 F.2d 1443, 1445-46, 24 U.S.P.Q.2d. 1443, 1444 (Fed. Cir. 1992).

Obviousness cannot be established by combining references without also providing objective evidence of the motivating force that would impel one skilled in the art to do what the patent applicant has done. *See In re Lee*, 61 U.S.P.Q.2d 1430, 277 F.3d 1338 (CAFC, 2002); *see also Ex Parte Levengood*, 28 U.S.P.Q.2d 1300, 1302 (Bd. Pat. App. & Inter. 1993)). Rejections on obviousness grounds cannot be sustained by mere conclusory

statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness. *KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385, 1396 (U.S. 2007).

If a proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there would be no motivation to make the proposed modification. *In re Gordon*, 733 F.2d 900, 221 USPQ 1125 (Fed. Cir. 1984). If the proposed modification or combination of the prior art would change the principle of operation of the prior art invention being modified, then the teachings of the references are not sufficient to render the claims *prima facie* obvious. *In re Ratti*, 270 F.2d 810, 123 USPQ 349 (C.C.P.A. 1959).

Appellants respectfully submit that the Examiner has not made a proper *prima facie* rejection under 35 U.S.C. §103(a), because the combination of prior art references cited fails to teach or suggest the present invention as recited in the independent claims and because it would not be obvious to combine the cited references. Additionally, it is well established that “[i]f an independent claim is non-obvious under 35 U.S.C. 103, then any claim depending therefrom is non-obvious.” *In re Fine*, 837 F.2d 1071, 1076, 5 USPQ2d 1596 (Fed. Cir. 1988).

1. Claims 52, 59-61, 63, 69-76, 78-91, 95, and 102 are patentable over WO 92/07581 in view of U.S. Patent No. 5,484,719.

The cited references do not teach each and every element of the presently claimed invention. Weiner is directed to a method for suppressing the immune response of a recipient mammal to non-self tissue from a donor mammal comprising: orally or enterally administering to said recipient mammal an agent in an amount effective for suppressing said immune response. The difference between Wiener and the present invention is that Wiener

teaches administration of animal tissue or cells such as splenocytes, or direct administration of the antigen. *See Weiner* at 7, line 24 to 8, line 23.

One difference between *Weiner* and the present invention is that nowhere in *Weiner et al.* is it suggested that oral administration of a transgenic plant is an alternative method of suppressing an immune response.

Lam et al. is directed to a vaccine produced in edible transgenic plants and then administered through the consumption of a part of the plant. *See, Lam et al.* at col. 3, lines 60-63. Persons of ordinary skill recognize that the purpose of a vaccine is the induction of an immune response. *Lam et al.* does not teach or suggest oral administration of transgenic plants for the suppression of an immune response. In fact, *Lam et al.* teaches that consumption of transgenic plant material from a plant expressing an antigen would produce the exact opposite effect desired in *Weiner*.

Thus, if a person of ordinary skill in the art were to read *Lam et al.* after *Weiner*, such a person would not contemplate modifying *Weiner* to arrive at the present invention as the expectation would be of a negative result.

The objectives of the two references are diametrically opposed and combining the references as proposed would change the principle of operation of *Lam et al.* Indeed, the objective of *Lam et al.* is the opposite of the objective of the present application. *Lam et al.* teaches that expression of antigens from viral, bacterial, or fungal antigens in a plant for a method of oral vaccination will have the effect of increasing the immune response to invading pathogens. In complete contrast, the present application is instead directed to the induction or oral tolerance with tolerogenic antigens with the object of suppressing the immune response, for example in situations of autoimmune disease or transplantation.

For this reason, the proposed combination of the cited references is *per se* non-obvious under the law. The mere fact that references can be combined or modified does not

render the resultant combination obvious unless the results would have been predictable to one of ordinary skill in the art. *KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385, 1396 (2007). In this case, the only reasonable prediction would be that modifying Weiner to use the transgenic plant techniques of Lam et al. would produce the opposite of the desired effect. If references taken in combination would produce a “seemingly inoperative device,” such references teach away from the combination and thus cannot serve as predicates for a prima facie case of obviousness. *McGinley v. Franklin Sports Inc.*, 60 USPQ2d 1001, 1010 (Fed. Cir. 2001) (*citing In re Sponnoble*, 405 F.2d 578, 587, 160 USPQ 237, 244 (CCPA 1969) (references teach away from combination if combination produces seemingly inoperative device)). Such a modification would render Weiner and Lam et al. both unsuitable for their intended purposes. It has long been recognized that if a proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification. *In re Gordon*, 733 F.2d 900, 221 USPQ 1125 (Fed. Cir. 1984). Moreover, as the principle of operation of Lam et al. and Weiner et al. are diametrically opposed, the combination of these references would necessarily require changing the mode of operation of Lam et al. It has long been recognized that if the proposed modification or combination of the prior art would change the principle of operation of the prior art invention being modified, then the teachings of the references are not sufficient to render the claims prima facie obvious. *In re Ratti*, 270 F.2d 810, 123 USPQ 349 (C.C.P.A. 1959).

The Examiner has argued, notably without citing supporting evidence, that these are “two sides of the same coin” and that “some immunologists refer to the induction of tolerance as the induction of a suppressive immune response.” *See* OFFICE ACTION DATED APRIL 14, 2009, at 10-11, bridging ¶¶. Respectfully, this opinion of the Examiner is incorrect. The induction of tolerance, as presently claimed, is very different from the induction of an

immune response, as is taught by Lam et al. If a person is tolerant to a specific antigen, this means that they mount little to no immune response to that antigen. This would be very desirable if the person is suffering from an autoimmune disease or has recently had an organ transplant. Lam et al. in contrast teaches vaccination for the prevention of disease. This would be very desirable if the person was interested in protecting himself from potential pathogens such as those that cause the flu. It would be completely undesirable for a person to be immune tolerant to such pathogens. Such a person would be completely incapable of mounting an immune response to the pathogen against which he was immune tolerant.

The Examiner has contended that one of ordinary skill in the art would believe that if viral, bacterial, and fungal antigens could be produced in a plant, then so could tolerogenic antigens. However, if the references were combined, the expected result could only be the opposite of the effect sought by the presently claimed invention.

Moreover, Applicants have presented evidence of secondary indicia of non-obviousness. For example, Dr. Jevnikar has been recognized in the art for his novel contributions in the field of immune research by the Kidney Foundation of Canada. See Exhibit 23. The Examiner discounts the recognition of Dr. Jevnikar's contribution as not addressing the specific invention. Applicants respectfully point out that the report of the award specifically refers to Dr. Jevnikar's "Novel expression and drug delivery systems for topical and oral delivery of proteins that modulate the body's immune response." This award is objective evidence of the recognition in the art of the novelty of Dr. Jevnikar's contributions in the subject matter of the presently claimed invention. Moreover, as objective evidence of commercial success and of addressing a long felt but unmet need, Dow AgroSciences Canada, Inc. is developing the technology for use with companion animals. See Exhibit 1. The presently claimed technology clearly addresses a long-felt but unmet need for commercial development in the field.

2. The Obviousness Rejection Should be Reversed

In view of at least the foregoing, Applicant respectfully submits that the Examiner has failed to establish a *prima facie* case of obviousness and the rejection of claims 52, 59-61, 63, 69-76, 78-91, 95, and 102 is improper. The combination of Weiner et al. and Lam et al. fails to teach or suggest each and every feature recited in claims 52, 59-61, 63, 69-76, 78-91, 95, and 102. Moreover, the combination of Lam et al. with Weiner would not have been predicted to be successful and would have rendered the method of Lam et al. unsuitable for its intended purpose and would have been contrary to the principle of operation disclosed in Lam et al. Therefore, Applicant respectfully submits that claims 52, 59-61, 63, 69-76, 78-91, 95, and 102 were improperly rejected under 35 U.S.C. § 103(a) and are patentable over the combination of Weiner et al. and Lam et al.

D. Claim Rejections Under The Judicially Created Doctrine of Nonstatutory Double Patenting

In the Office Action dated April 14, 2009, claims 52, 59, and 60 were rejected for allegedly overlapping with claims 17-20, 34, and 53 of U.S. Patent Application No. 11/815,359 (published as US 2008-0253991 attached hereto as Exhibit 24; copy of currently pending claims in the '359 application attached as Exhibit 25).

A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). *See, e.g., In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); and *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985). A two-way test is to be applied when the applicant could not have filed the claims in a single application and there is administrative delay. M.P.E.P. § 804; *In re Berg*, 46 USPQ2d

1226 (Fed. Cir. 1998) (the two-way exception applies when the applicant could not avoid separate filings, and if the PTO controlled the rates of prosecution to cause the later filed species claims to issue before the claims for a genus in an earlier application).

The rejection is necessarily provisional as the claims of the '359 application have not issued. The correct course of action in this case would be to allow the present claims to issue and then determine whether a double patenting rejection would be appropriate in the later filed application. M.P.E.P. § 804.

1. Claims 52, 59, and 60 are patentable over claims 17-20, 34, and 53 of U.S. Patent Application No. 11/815,359.

The claims of the '359 application are directed to a separately patentable invention. Claims 17-20 of the '359 application depend ultimately from claim 1. Independent claims 1, 34, and 53 of the '359 application all recite methods directed to treatment or reversal of Type I diabetes or of a pre-Type I diabetic mammal, human, or animal. Each of the claimed methods recites that autoantigen composition, which may be in the form of transgenic plant material, is administered to the mammal, human, or animal. However, as recited in the cited claims, the methods comprise administration of further therapies to the mammal, human, or animal, which are not mentioned in the present claims. For example, claim 1 recites "administering anti-T cell therapy to said mammal." Claim 34 recites "administering an effective immunosuppressive dose of anti-T cell antibodies to said humans." Claim 53 recites "administering a therapeutically effective amount of anti-CD3 monoclonal antibody to said human or animal." Since none of these features are found anywhere in the present claims, the claims cannot be said to be directed to the same invention.

The '359 application is a later filed application. The present claims have been held up by the PTO due to the rejections appealed above, which must be reversed for the reasons set forth above, and the need for this appeal. Thus, this case requires application of a two-way

test. Applicants submit that the species recited in the claims of the '359 application would not have been obvious over the presently appealed claims. A two-way test is appropriate in this case, because the present claims should have been allowed to issue long ago. *See* M.P.E.P. § 804; *In re Berg*, 46 USPQ2d 1226 (Fed. Cir. 1998) (the two-way exception applies when the applicant could not avoid separate filings, and if the PTO controlled the rates of prosecution to cause the later filed species claims to issue before the claims for a genus in an earlier application).

The statement of the rejection fails to set forth a prima facie case of obviousness. Moreover, the Examiner has not correctly applied a two-way test. Accordingly, reversal of the double patenting rejection is appropriate and is respectfully requested.

E. Conclusion

Each of the rejections raised in the Office Action being in error, it is therefore respectfully requested that this Honourable Board reverse the Examiner's decision in this case and indicate the allowability of claims 52, 59-61, 63, 69-91, 95, and 102.

VIII. Claims Appendix

See attached Claims Appendix for a copy of the claims involved in the appeal.

IX. Evidence Appendix

See attached Evidence Appendix for copies of evidence relied upon by Appellant.

X. Related Proceedings Appendix

There being no decisions of the Board or a court in any proceedings identified in Section II, *supra*, the Related Proceedings Appendix has been omitted.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

Date December 14, 2009

By: /Christopher L. North/
Christopher L. North
Registration No. 50433

Customer No. 21839
703 836 6620

VIII. CLAIMS APPENDIX

The Appealed Claims

52. A method for suppressing or reducing the immune response of a mammal to an antigen comprising:

orally or enterally administering to the mammal an effective immune suppressive dose of a plant tissue or a partially purified plant tissue extract containing said antigen or an immunosuppressive fragment thereof, said plant tissue or partially purified plant tissue extract being obtained from a transgenic plant expressing said antigen or immunosuppressive fragment thereof.

59. The method of claim 52, wherein the plant tissue or partially purified plant tissue extract is selected from the group consisting of at least one plant part, an extract of total plant protein, and a partially purified plant protein preparation.

60. The method of claim 52, wherein the plant tissue or partially purified plant tissue extract is from at least one plant part selected from the group consisting of leaves, stems, seeds and tubers.

61. The method of claim 52, wherein the transgenic plant is transformed with a DNA construct for transforming a plant, said construct comprising a Cauliflower Mosaic Virus Ehn-35S promoter operably linked to a DNA coding sequence and further comprising a termination sequence in proper reading frame with the DNA coding sequence, wherein the termination sequence is a nopaline synthase termination sequence and the DNA coding sequence encodes the antigen.

63. A pharmaceutical composition for suppressing or reducing the immune response of a mammal to an antigen comprising:

an oral or enteral dosage form comprising an effective immunosuppressive dose of a plant tissue or partially purified plant tissue extract containing said antigen or an immunosuppressive fragment thereof and a pharmaceutically acceptable carrier, said plant tissue or partially purified plant tissue extract being obtained from a transgenic plant expressing said antigen or immunosuppressive fragment thereof.

69. The composition of claim 63, wherein the transgenic plant is selected from the group consisting of potato, tomato, alfalfa, canola and low alkaloid tobacco.

70. The composition of claim 63, wherein the plant tissue or partially purified plant tissue extract is from at least one plant part selected from the group consisting of leaves, stems, seeds and tubers.

71. The composition of claim 63, wherein the transgenic plant is transformed with a DNA construct for transforming a plant, said construct comprising a Cauliflower Mosaic Virus Ehn-35S promoter operably linked to a DNA coding sequence and further comprising a termination sequence in proper reading frame with the DNA coding sequence, wherein the termination sequence is a nopaline synthase termination sequence and the DNA coding sequence encodes the antigen.

72. The method of claim 52, wherein the antigen is a mammalian transplantation antigen.

73. The method of claim 72, wherein the transplantation antigen is a human Major Histocompatibility Complex (MHC) protein.

74. The method of claim 73, wherein the MHC protein is selected from the group consisting of an MHC class I protein, an MHC class II protein, an MHC class IIa chain and an MHC class IIb chain.

75. The method of claim 72, wherein the transgenic plant is selected from the group consisting of potato, tomato, alfalfa, canola and low alkaloid tobacco.

76. The method of claim 72, wherein the plant tissue or partially purified plant tissue extract is from at least one plant part selected from the group consisting of leaves, stems, seeds and tubers.

77. The method of claim 72, wherein the mammal is a human.

78. The composition of claim 63, wherein the antigen is a mammalian transplantation antigen.

79. The composition of claim 78, wherein the transplantation antigen is a human Major Histocompatibility Complex (MHC) protein.

80. The composition of claim 78, wherein the MHC protein is selected from the group consisting of an MHC class I protein, an MHC class II protein, an MHC class IIa chain and an MHC class IIb chain.

81. The composition of claim 78, wherein the transgenic plant is selected from the group consisting of potato, tomato, alfalfa, canola and low alkaloid tobacco.

82. The composition of claim 78, wherein the plant tissue or partially purified plant tissue extract is from at least one plant part selected from the group consisting of leaves, stems, seeds and tubers.

83. The composition of claim 78, wherein the mammal is a human.

84. A method for suppressing the rejection of engrafted donor tissue in a recipient mammal comprising orally or enterally administering to the mammal an effective immunosuppressive dose of a plant tissue or a partially purified plant tissue extract containing a transplantation antigen of said donor tissue or an immunosuppressive fragment thereof, said plant tissue or partially purified plant tissue extract being obtained from a transgenic plant expressing said transplantation antigen or immunosuppressive fragment thereof.

85. The method of claim 84, wherein the transplantation antigen is an MHC protein.

86. The method of claim 85, wherein the MHC protein is selected from the group consisting of an MHC class I protein, an MHC class II protein, an MHC class II chain and an MHC class II chain.

87. The method of claim 84, wherein the transgenic plant is selected from the group consisting of potato, tomato, alfalfa, canola and low alkaloid tobacco.

88. A transgenic plant comprising a plant expressing a recombinant mammalian transplantation antigen.

89. The transgenic plant of claim 88, wherein the transplantation antigen is a human Major Histocompatibility Complex (MHC) protein.

90. The transgenic plant of claim 89, wherein the MHC protein is selected from the group consisting of an MHC class I protein, an MHC class II protein, an MHC class II chain and an MHC class IIb chain.

91. The transgenic plant of claim 88, wherein the plant is selected from the group consisting of potato, tomato, alfalfa, canola, and low alkaloid tobacco.

95. An edible plant material comprising a plant tissue or partially purified plant tissue extract obtained from a transgenic plant of claim 88.

102. A method for suppressing or reducing the immune response of a mammal to an MHC Class II protein comprising:

orally or enterally administering to the mammal an effective amount of a plant tissue or a partially purified plant tissue extract obtained from a transgenic plant expressing an MHC Class II protein or an immunosuppressive fragment thereof.

IX. EVIDENCE APPENDIX

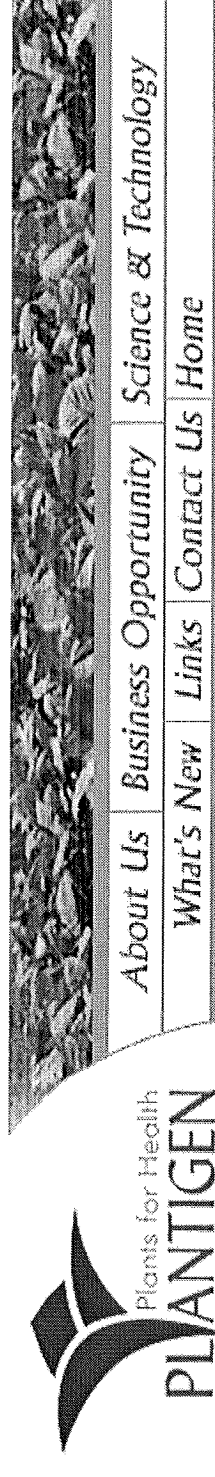
Exhibit No.	Description	Where cited
Exhibit 1	Plantigen, Dow AgroSciences Press Release	Cited by Applicants in Response dated October 9, 2007
Exhibit 2	1999 Jevnikar Declaration	Filed by Applicants
Exhibit 3	2000 Jevnikar Declaration	Filed by Applicants
Exhibit 4	2005 Jevnikar Declaration	Filed by Applicants
Exhibit 5	Ma et al., 1997	Cited by Applicants in IDS filed December 7, 2001
Exhibit 6	Marketletter, 1999	Cited by Examiner on February 10, 2004
Exhibit 7	Pozzolli et al., 2000	Cited by Examiner on April 14, 2009
Exhibit 8	Skylar et al., 2005	Cited by Examiner on April 14, 2009
Exhibit 9	Dong et al., 1999	Cited by Examiner on April 14, 2009
Exhibit 10	WO 02/053092	Cited by Examiner on February 10, 2004
Exhibit 11	Goodnow, 2001	Cited by Examiner on February 10, 2004
Exhibit 12	Kraus and Mayer, 2005	Cited by Examiner on April 14, 2009
Exhibit 13	Bell et al., 2008	Cited by Examiner on April 14, 2009
Exhibit 14	Husby et al., 1994	Cited in Declaration of Anthony M. Jevnikar submitted February 8, 2005
Exhibit 15	Moldoveanu et al., 2004	Cited by Examiner on April 18, 2005

Exhibit 16	McKown et al., 2000	Cited in Declaration of Anthony M. Jevnikar submitted February 8, 2005
Exhibit 17	McKown et al., 1999	Cited by Examiner on April 18, 2005
Exhibit 18	Carbone et al., 2004	Cited by Examiner on April 18, 2005
Exhibit 19	Womer et al., 2008	Cited by Applicants in Response dated February 3, 2009
Exhibit 20	Ergun-Longmire et al., 2004	Cited by Applicants in IDS filed February 3, 2009
Exhibit 21	WO 92/07581	Cited by Applicants in IDS filed December 7, 2001
Exhibit 22	U.S. Patent No. 5,788,968	U.S. Patent corresponding to WO 92/07581 cited by Applicants in IDS filed December 7, 2001
Exhibit 23	The Kidney Foundation of Canada	Cited by Applicants in Response dated October 9, 2007
Exhibit 24	US 2008-0253991	Publication of U.S. Patent Application No. 11/815,359 cited in Office Action dated April 14, 2009
Exhibit 25	Copy of currently pending claims in U.S. Patent Application No. 11/815,359	U.S. Patent Application No. 11/815,359 cited in Office Action dated April 14, 2009

X. RELATED PROCEEDINGS APPENDIX

There being no decisions of the Board or a court in any proceedings identified in Section II, *supra*, the Related Proceedings Appendix has been omitted.

EXHIBIT 1



Plantigen, Dow AgroSciences Announce Collaboration

FOR IMMEDIATE RELEASE:

London, Ontario (June 21, 2003)

(London, ON) Plantigen Inc. announced today it has signed a collaborative research agreement with Calgary-based Dow AgroSciences Canada Inc. The research agreement is focused on plant-made antigens that prevent auto-immune disease in companion animals. The collaboration will build on Plantigen's intellectual property and expertise in immune modulation using mammalian proteins derived from plants.

"This collaboration between Plantigen Inc. and Dow AgroSciences, exemplifies our commitment to research collaborations, partnerships, and driving towards product innovation," said Butch Mercer, Global Business Leader, Animal Health & Nutrition for Dow AgroSciences. "Plantigen's unique technology and their medical 'hands-on' clinical expertise in the field of auto-immune diseases, coupled with Dow AgroSciences' plant biotechnology expertise, creates the critical mass of technologies required to bring new solutions to address debilitating health conditions for companion animals."

"We see this collaboration as a unique opportunity to demonstrate the promise of this technology in delivering safe, efficacious and cost-effective doses of mammalian proteins, in order to combat serious diseases such as Diabetes, Crohns and other disorders of the immune system," said Dr. Anthony Jevnikar, Chief Scientific Officer for Plantigen Inc. "Together with the strength that Dow AgroSciences offers in agriculture and plant technology, we will be able to leverage our medical expertise to make this promise a reality."

ABOUT PLANTIGEN

Plantigen Inc. is a discovery company focused on the identification and development of plant-derived biopharmaceuticals for the prevention and treatment of human disease. The company is a spin-off of the Lawson Health Research Institute, the research arm of the founding hospital in London, Ontario, Canada. Plantigen is developing products using genetically enhanced plants - ORAmmune™ and PHYTOkines™ to combat immune system disorders. These products are in their pre-clinical stage of development.

Please visit www.plantigen.com

ABOUT DOW AGROSCIENCES

Dow AgroSciences Canada Inc. is a subsidiary of Dow AgroSciences LLC, based in Indianapolis, Indiana, USA. Dow AgroSciences is a global leader in providing pest management and biotechnology products that improve the quality and quantity of the earth's food supply and contribute to the safety, health and quality of life of the world's growing population. Dow AgroSciences has approximately 6,000 people in more than 50 countries dedicated to its business, and has worldwide sales of more than US \$2.7 billion. Dow AgroSciences is an indirect wholly owned subsidiary of The Dow Chemical Company. For more information about Dow AgroSciences, visit www.dowagro.com

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EXHIBIT 2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of)	
Jevnikar et al.)	Examiner: M. Lubet
Serial No. 08/617,874)	
)	Group Art Unit: 1644
Filed: May 21, 1996)	
)	
For: METHODS AND PRODUCTS)	
FOR CONTROLLING IMMUNE)	Date: September 27, 1999
RESPONSES IN MAMMALS)	

DECLARATION OF ANTHONY M. JEVNIKAR

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Anthony M. Jevnikar, do hereby declare and say as follows:

1. I am presently the Director of the Department of Transplantation Nephrology, London Health Sciences Centre, in London, Ontario, Canada. I am also Associate Professor in the Faculty of Medicine, Department of Microbiology and Immunology, in the University of Western Ontario. A copy of my curriculum vitae is attached as Exhibit 1 to this Declaration.

2. I am a co-inventor named in the above-identified application and have read and understood the Office Action mailed on April 10, 1998 and the Advisory Action mailed on December 11, 1998.

3. The Examiner has rejected claims 19-21, 28, 31-36, 42 and 53-55 as unpatentable over U.S.P. 5,484,719 (Lam et al.), in view of U.S.P. 5,475,086 (Tobin et al.). The Examiner has also rejected claims 1, 5-10, 19-21, 28, 31-36, 41-42 and

53-55 as unpatentable over U.S.P. 5,643,868 (Weinier et al.) and Zhang et al. (P.N.A.S., 88, 10253-10256) in view of U.S.P. 5,484,719 (Lam et al.) and U.S.P. 5,475,086 (Tobin et al.).

4. Lam et al. discloses the production of oral vaccines by expressing antigens characteristic of pathogenic organisms such as hepatitis B virus in plants. Oral administration of such transgenic plants gave stimulation of an immune reaction against the expressed antigen in the subject receiving the vaccine. Stimulation of an immune response is the opposite of inducing immune tolerance, as achieved by the subject invention.

5. It was known prior to the priority date of the present application, September 21, 1993, that plant proteins were glycosylated differently from mammalian proteins. If plant-expressed foreign proteins were also glycosylated differently from normal, or were otherwise slightly altered, this might potentially increase their antigenicity. Such a change would be beneficial if one's aim, as in Lam et al., was to stimulate an immune reaction. It was not known at that date, and it was not predictable, whether the glycosylation pattern of mammalian antigenic proteins would be affected by expression in a plant system or whether an antigen so produced would be effective to induce oral tolerance in a mammal.

6. I have reviewed U.S.P. 5,475,086 (Tobin et al.) and its teachings, at columns 8 to 10, regarding therapy of autoimmune diabetes. In column 8, lines 31 to 55 and column 9, lines 1 to 25, the authors describe the identification of a polypeptide region common to the amino acid sequences of human GAD₆₅, human GAD₆₇ and the P2-C protein of coxsackie virus. They postulate that "molecular mimicry" plays a role in the development of autoimmune diabetes, in that after infection with coxsackie virus and activation of T cells to recognise this common polypeptide region, the activated T cells then give rise to an immune response mounted against GAD proteins in the subject's β cells, thus destroying the β cells and causing

diabetes. They suggest that treatment with a polypeptide having the sequence of this common region will block this undesired immune response and prevent disease development (column 9, lines 26 to 46).

This teaching does not suggest that oral administration of plant-expressed, transgenic GAD protein, or even of GAD protein, could prevent the development of autoimmune diabetes.

7. An alternative therapy is proposed at column 9, line 47 to column 10, line 22. The authors suggest stimulation of T-suppressor cells "to restore self-recognition" and ameliorate the disease, by administering a bi-specific antibody, specific both for an epitope of the autoimmune antigen and for an epitope present on the CD8⁺ receptor.

Again, this teaching in no way suggests that the oral administration of a plant material containing plant-expressed GAD will induce oral immune tolerance, which is not dependent on CD8⁺ T cells, nor on the ability to cross-link GAD peptides, within or without MHC proteins which might contain them, to T cell receptors. The production of antibodies to GAD is not required for the induction of oral immune tolerance by feeding plant material containing GAD.

8. Finally, it is suggested that polypeptide analogs can be designed "which will compete for recognition of self-antigens at the level of antigen presentation", but "will not activate disease-causing T-helper cells" (column 9, lines 60 to 67). I interpret this to be a suggestion that one can devise analogs of the common polypeptide sequence described by these authors, as discussed in paragraph 9 above, by modification of the amino acid sequences shown in Table 1, as further discussed through to column 11, line 19.

9. None of the therapeutic approaches proposed by Tobin et al., as discussed in paragraphs 6 to 8 above, would, if followed, have led to any appreciation that autoimmune diabetes could be prevented in a susceptible subject by oral

administration of plant material containing plant-expressed GAD protein, as described in the present application. I consider that Tobin et al. teaches away from the present invention, in that the therapeutic approaches suggested would not, in my opinion, lead to oral immune tolerance to an autoantigen such as GAD.

10. I do not believe that the teachings of Lam et al. regarding stimulation of a subject's immune response by administration of plant-produced antigens can be combined with the teachings of Tobin et al., as discussed above, to arrive at the invention described in the present invention.

11. Weiner et al. and Zhang et al. described feeding a native mammalian autoantigen, insulin, to mammals susceptible to autoimmune diabetes to produce oral tolerance to the autoantigen and suppression of the development of the disease.

12. Prior to the work of the present inventors, no one had suggested suppressing or reducing the immune response of a mammal to a mammalian transplantation antigen or autoantigen by feeding to the mammal a plant material obtained from a transgenic plant expressing the relevant transplantation antigen or autoantigen. As noted above, in paragraph 5, it was not known prior to the priority date of this application, and it was not predictable, whether a mammalian antigenic protein would be produced with complete fidelity in a plant-expression system, for example with respect to glycosylation patterns, or whether an antigen so produced would be effective to induce oral tolerance in a mammal. There is nothing in the teachings of Weiner et al. or Zhang et al. to give any guidance in this regard.

13. The present inventors have found that plant material obtained from a transgenic plant expressing a mammalian autoantigen can be used to produce oral immune tolerance, as described in the application and further described in Nature

Medicine (1997), v. 3, p. 739, a copy of which was filed with Applicant's response dated January 5, 1998.

14. Furthermore, the inventors have found unexpectedly that plant material containing plant-expressed mouse GAD protein stimulated a greater proliferative response of GAD-primed T cells than highly purified recombinant mouse GAD expressed in E.coli. This study is described in greater detail in Exhibit 2 attached. These findings indicate that plant material containing plant-expressed transgenic GAD gave enhanced T cell activation, which is a pre-requisite step in the induction of immune tolerance, including oral tolerance.

15. Such enhancement may be related to altered glycosylation of the antigen when expressed in plants; it has been shown that the glycosylation of an allergen influence the affinity of antibodies against that allergen (Batanero et al., (1994), Molecular Immunology, 31, 31). Similarly, the processing, transport and binding of antigen fragments to MHC molecules on antigen-presenting cells, which plays a central role in oral tolerance, will be affected by the glycosylation pattern of the antigen.

16. Additionally, oral administration of plant material obtained from a transgenic plant expressing a mammalian transplantation antigen or autoantigen results in administration not only of the recombinant antigen but also of additional plant components which may assist in the induction of oral immune tolerance. For example, plant lectins are bound to nucleated cells of the gut and lectins are known to arrive intact in the small intestine, where there are lymphoid cells involved in the induction of oral tolerance.


Lectins bound to gut cells can be endocytosed by the gut epithelium, where they can have a direct effect on the epithelial cells and, by acting as growth factors, can affect the immune response (Pusztai, (1993), Eur. J. Clin. Nutrition, 47, 691). As one example, Vehmeyer et al.,(1998), Eur. J. Haematol., 60, 16, has recently

shown that the plant lectin VAA-1 binds to haematopoietic cells, including CD34+ progenitor cells which are represented in the gut, and has a co-stimulatory effect on proliferation of such cells in humans. Many other lectins are known to exist in plant cells.

As noted above, T cell activation is required in order to produce oral tolerance.

17. The teachings of Lam et al. and Tobin et al. cannot, in my opinion, be combined to arrive at the present invention, as discussed in paragraph 1D above; the teachings of Weiner et al. and Zhang et al. regarding use of a native mammalian autoantigen do not supplement the teachings of Lam and Tobin so as to arrive at an appreciation that plant material containing plant-expressed mammalian antigens could be administered orally to produce immune tolerance.

18. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardise the validity of the application or any patent issued thereon.


Anthony M. Jeynikar

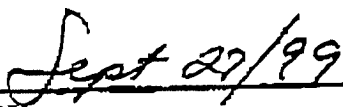

Date

EXHIBIT 1

EXHIBIT 1

CURRICULUM VITAE

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July 1991-June 1996 Assistant Professor Department of Medicine
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1971-76 Honours B.Sc. Microbiology, University of Western Ontario (UWO)
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AWARDS:

1980 Alpha Omega Alpha Medical Society
1981 F. R. Eccles Scholarship, UWO
1981 Alpha Kappa Kappa Gold Medal, UWO
1988-91 Medical Research Council of Canada Research Fellowship

1991-93 Kidney Foundation of Canada Scholarship
1992 Canadian Society of Nephrology-Upjohn Young Investigator Award
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POST GRADUATE EDUCATION:

1981-82 Rotating Internship, Victoria Hospital, London, Canada
1982-83 General Medical Resident (R1), UWO
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1988-91 Immunology and Molecular Biology Research Fellow
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LICENSING EXAMINATIONS:

1981 Medical Council of Canada, Examinations, Reg. #54165
1981 College of Physicians & Surgeons of Ontario, Reg. #50851
1986 F.R.C.P. (C) Internal Medicine, Reg. #315076

MEMBERSHIPS:

1980 Ontario Medical Association
1980 Canadian Medical Association
1986 Canadian Society of Nephrology
1990 Canadian Transplantation Society
1991 International Society of Nephrology
1992 American Society of Nephrology
1994 Canadian Society for Immunology
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COMMITTEES AND TEACHING:

1981-82 Postgraduate Medical Education Committee, Victoria Hospital
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CURRENT RESEARCH INTERESTS:

1. Effect of major histocompatibility complex molecule deletion in autoimmunity.
2. Role of TGF β and apoptosis in lupus nephritis
3. The use of transgenic plants expressing mammalian proteins in inducing oral tolerance.
4. The role of CD45 in renal transplant rejection.

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PUBLICATIONS:

1. Jevnikar AM: Characteristics of immunologically reactive cells from human allograft recipients M.Sc. thesis, UWO, 1977.
2. Stiller CR, Sinclair NR StC, McGirr D, Jevnikar AM, Ulan RA: Diagnostic and prognostic value of donor specific post transplant immune responses; Clinical correlates and in vitro variables: *Transplant. Proc.* 1978;10:525.
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4. Jevnikar AM, Finnie KJC, Dennis B, Plummer DT, Avila A, Linton AL: Nephrotoxicity of high and low osmolality contrast media: *Nephron* 1988;48(4):300.
5. Jevnikar AM, Petric R, Holub BJ, Philbrick D, Clark WF: Effect of cyclosporine on plasma lipids and modification with dietary fish oil: *Transplantation* 1988;46(5):722.
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2. Parbtani A, Jiang JF, Mukherjee R, Yin Z-Q, Chakrabarti S, Garcia B, Zhong R, Jevnikar AM: Protective effect of donor Fas or Fas ligand deficiency in renal allograft rejection. Submitted.

3. Mukherjee R, Parbtani A, Yin Z-Q, Chakrabarti S, Jevnikar AM: Reducing TGF β augments apoptosis and improves nephritis in MRL-*lpr* mice. Submitted.

4. McLaughlin K, Rizkalla KS, Adams PC, Grant DR, Wall WJ, Jevnikar AM, Marotta P: Hepatitis C infection is not associated with an increased risk of acute liver allograft rejection. Submitted.

PATENTS:

Inventor: Methods And Products For Controlling Immune Responses In Mammals. European Patent Application No. 94928233.9 (Regional Phase of PCT/CA94/00530).

EXHIBIT 2

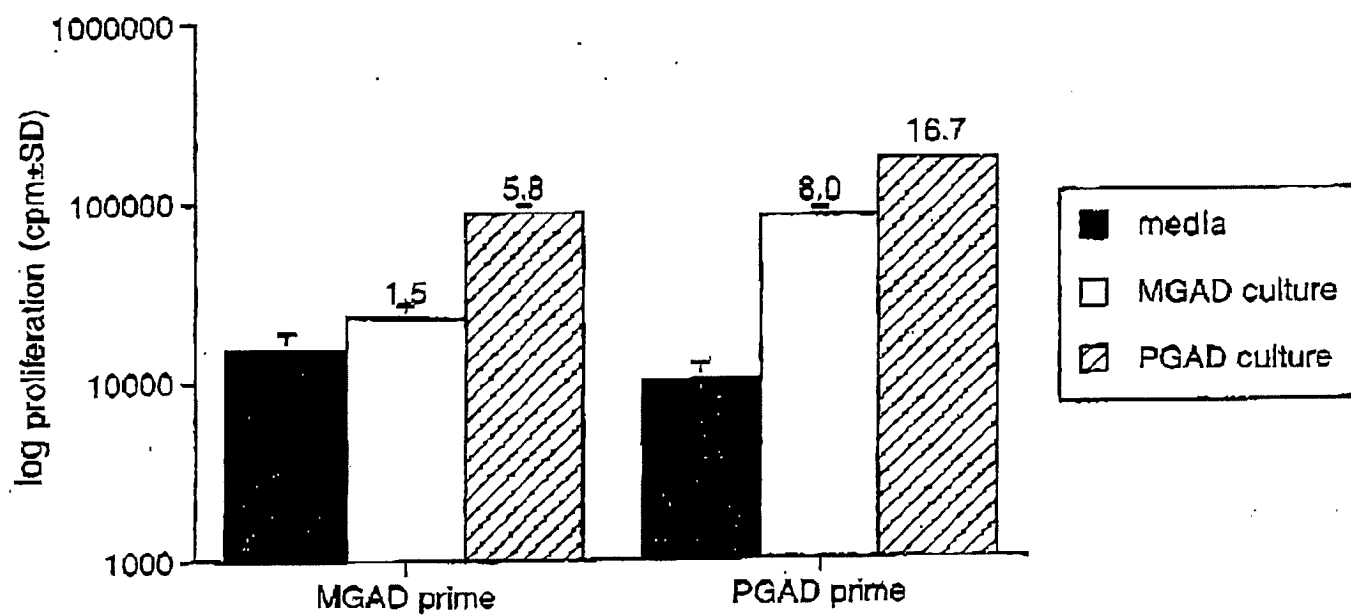
EXHIBIT 2 - page 1 of 2

T cell proliferation assay. T cell responses to transgenic plant GAD protein were assessed using lymphocytes from GAD-primed non diabetic NOD/Lt mice. Mice were used from an established colony and were given standard food and water ad libitum.

Mouse GAD67 was prepared recombinantly in E.coli and purified (MGAD). Mouse GAD67 was also expressed in transgenic tobacco, as described in this application, and a plant extract containing expressed GAD67 (PGAD) was prepared by crushing leaves in buffer in a mortar and pestle and removing plant debris by centrifugation. Total protein concentration was determined by spectrophotometry.

50µg of purified MGAD in 50µl PBS or 500µg of PGAD in 50µl plant extract was emulsified with an equal volume of incomplete Freund's adjuvant (IFA, Sigma Chemical Co., MO) and injected into each hind foot pad of 6 to 8 week old female non-diabetic NOD mice. IFA with PBS alone was used as control. The mice were killed after 10 days, popliteal lymph nodes were removed and a single cell suspension was prepared. The cells were then cultured in 96-well flat bottom plates (Becton Dickinson, New Jersey) at 2×10^5 cells/well in RPMI (Biowhittaker, Maryland) supplemented with 10% FCS (GIBCO, Grand Island, NY), 10 mM HEPES, 5×10^{-5} M 2-ME and 1 U/ml Penicillin-Streptomycin. Cultures were stimulated with increasing concentrations of MGAD (1-100 µg/ml), or PGAD (1 - 500 µg/ml), incubated for 72 hours, pulsed with 3H- thymidine (1µCi/well; DuPont-NEN, Boston, MA), harvested on glass fiber filter 16 h later and counted in a liquid scintillation counter (LKB Instruments, Gaithersburg, MD). The ratio of cpm of stimulated cells to media controls was expressed as stimulation indices (SI).

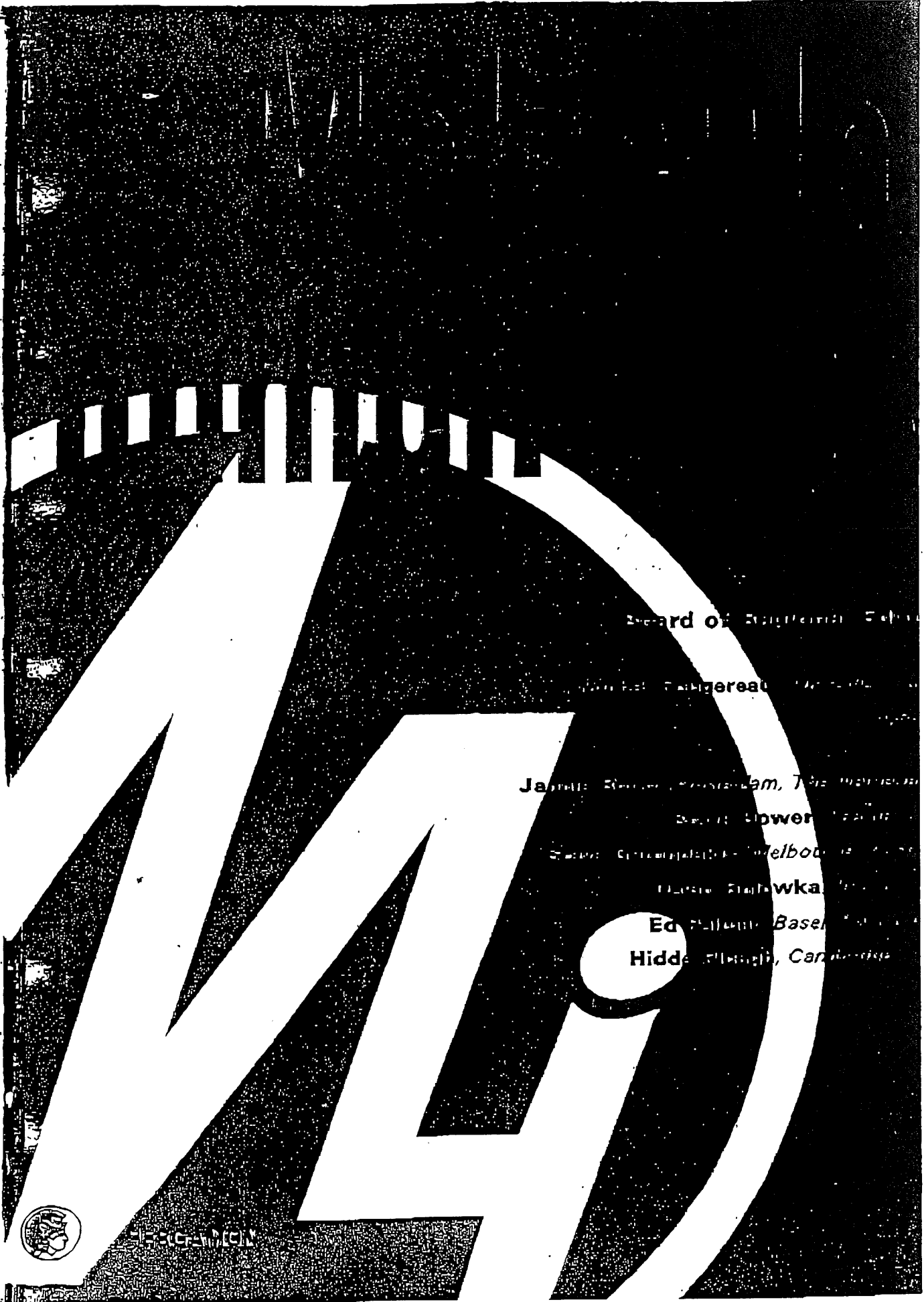
The results are shown in the attached Figure, which shows that priming of NOD mice with either plant derived GAD (PGAD) or highly purified recombinant GAD (MGAD) caused lymph node cell proliferation in response to either MGAD or PGAD protein *in vitro*. The highest proliferation was observed in response to PGAD, using T cells primed *in vivo* with PGAD (stimulation index (SI)= 16.7), and to MGAD using T cells primed with PGAD *in vivo* (SI= 8.0), showing that crude plant extracts are immunoreactive *in vivo* and actually enhance T cell proliferation. This effect was evident in dose response studies in which T cells were exposed to equivalent amounts of GAD67 in plant extract, based on determination of GAD67 in the soluble fraction of total plant protein being in the range of 0.4%.



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Review

Dietary lectins are metabolic signals for the gut and modulate immune and hormone functions

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Although it is common knowledge that some dietary lectins can adversely affect the growth and health of young animals and that, therefore, lectins are implicated in nutritional disorders of the digestive system, it has not been rigorously established that findings with animals are also directly applicable to humans. However, because the glycosylation state of the human gut is basically similar to that of higher animals, it may be confidently predicted that the effects of dietary lectins will have similarities in both humans and animals. The more recent, but not generally appreciated realization that lectins also have many beneficial effects on the gut and metabolism of animals makes the exploration of these for possible use in medical-clinical practice even more attractive. Most lectins in our diet are resistant to breakdown during gut passage and are bound and endocytosed by epithelial cells. These lectins are powerful exogenous growth factors for the small intestine, can induce dramatic shifts in its bacterial flora and interfere with its hormone secretion. In addition, lectins which are transported across the gut wall into the systemic circulation can modulate the body's hormone balance, metabolism and health. Although these physiological effects are mediated or reinforced by immune responses, they are primarily the result of the specific chemical reactivity of lectins with cell surface receptors of the gut. Clearly, as the interactions between lectins and the gut are predictable and may be manipulated to our advantage, the exciting scientific challenge is now to explore the possible transfer of the existing knowledge gained from animal experimentation to medical-clinical practice.

Lectins are carbohydrate-binding (glyco)proteins of non-immune origin capable of specific recognition of, and reversible binding to, carbohydrates without altering their covalent structure (Kocourek & Horejsi, 1983). They are essential and omnipresent plant constituents and as a major part of our food is of plant origin, appreciable amounts of lectins are ingested daily. For example, Nachbar & Oppenheim (1980) found that 30% of fresh and processed food had significant haemagglutinating activity. They also found evidence for the presence of lectins in 53 edible plants. These observations have been confirmed and extended by the

finding of a correlation between lectin activity and antinutritional effects (Grant *et al.*, 1983).

During evolution our gut has been regularly exposed to lectins, which must therefore have played an important part in the development of the digestive system. As experience has taught us which foodstuffs need to be avoided, toxic plants have been eliminated from our diet. However, it is more difficult to associate the antinutritional, mild allergic or other subclinical symptoms with the food we eat or with a particular component of it. As some lectins are at least partially heat stable and most survive the passage through the gut in functionally and

immunologically intact form. Interactions with potentially serious consequences between lectins in the diet and the gut may occur (Pusztai, 1989a). Although it is known, mainly from animal studies, that dietary lectins can damage the gut and that this may lead to various nutritional disorders, it is not generally appreciated that lectins can also have beneficial effects and that these may find uses in medical-clinical practice. Thus in the present review, in addition to a general description of the main physiological effects of lectins on the gut, metabolism and health established mainly from studies on experimental animals, there will be a special emphasis on the present and possible future trends for the use of lectins in medicine.

Local effects

Resistance to proteolysis

Most plant lectins (phytohaemagglutinins; PHAs) are resistant to breakdown during passage through the gut and this is the basis for their striking physiological and nutritional effects (Pusztai, 1991; Pusztai *et al.*, 1991a). Lectins, such as those from kidney bean (*Phaseolus vulgaris*), PHA, jackbean (*Canavalia ensiformis*), con A, and the agglutinin from snowdrop bulbs (*Galanthus nivalis*) (GNA), survive almost quantitatively. Even with less resistant lectins the proportion surviving was at least 20% of that fed (Pusztai, 1991).

Binding of lectins by the small intestine

Binding to membrane glycans of epithelial cells of the small intestine is a necessary prerequisite for a lectin to be physiologically active in the gut. However, as the distribution of glycosylated receptors varies at different locations, the binding and endocytosis of the agglutinins by the epithelial cells may also vary in the different functional parts of the gut. The extent of binding varies from practically none to complete, and is correlated with the effectiveness of the lectins as growth factors for the gut (Table 1). Thus, lectins which have slight reactivity with the brush border epithelium, such as those from peas or broad beans, do not stimulate the growth of the small bowel (Pusztai *et al.*, 1990a). The moderately reactive tomato lectin (Kilpatrick *et al.*, 1985) or the wheat germ agglutinin, both of which are specific for

Table 1. Effect of lectins and trypsin inhibitor on the small intestine

	Binding	Small intestine		
		Weight	Crypt length	Polyamines
PHA	++++	206	216	188
RPA; <i>Robinia</i>	++++	183	175	153
SNA-II; elder	+++	125	134	150
SBA; <i>soya</i>	++	121	158	115
WGA; wheat germ	++	145	160	129
DSA; <i>Datura</i>	++	115	109	130
UDA; nettle	++	117	126	120
SNA-I; elder	+	111	114	138
MAA; <i>Maackia</i>	±	112	95	94
STI; trypsin inhibitor	—	108	110	121

Binding to the small intestine is expressed on an arbitrary scale of +++++ (strongest) to — (no binding), as assessed from the intensity of peroxidase-antiperoxidase staining after reacting the sections with the appropriate anti-lectin antibodies. The weight, crypt length and total polyamine content of the small intestine of rats fed with diets containing 0.7% of lectins or trypsin inhibitor for 10 days are expressed relative to those of the control diet (taken as 100).

N-acetylglucosamine, are more effective growth stimulants as they are bound both by the cells of the villus tip and the lower parts of the villi (Fig. 1a). The most powerful growth factors for the gut are those lectins, such as PHA and SBA, which are strongly bound to and endocytosed extensively by villus tip cells (Fig. 1b; Pusztai *et al.*, 1990a). These induce hyperplastic growth by stimulating crypt cell proliferation. Furthermore, since a proportion of the endocytosed lectins is transported into the blood circulation, they also have powerful systemic effects and induce metabolic changes in the body.

Lectin-binding by the gut or other tissues can have potentially important applications in clinical practice. Thus, it is possible that low concentrations of non-toxic lectins (tomato lectin, wheat germ agglutinin, etc.) may, in future, be used safely as growth stimulants in small intestinal hypoplasia induced by total parenteral feeding, resection or other gut lesions. Furthermore, as changes in surface glycosylation can be correlated with various disease states of the alimentary tract and other tissues, particularly as early markers in malign-



Fig. 1. (a) Binding and endocytosis of wheat germ agglutinin and (b) kidney bean lectin, PHA, by enterocytes of rat small intestine. Specific immunoreactive PAP (peroxidase-antiperoxidase) staining of sections from the jejunum of rats fed diets containing (a) wheat germ agglutinin or (b) PHA for 10 days. Formalin-fixed sections were reacted with the respective monospecific rabbit anti-lectin antibodies. Treatments with second antibody (PAP) and 3,3'-diaminobenzidine were followed by counterstaining with haematoxylin. Bar = 100 μ m.

nancy, labelled lectins are already routinely employed for the staining of biopsies as histochemical reagents (Stevenson, Griffith & Mills, 1986; for a review see Spicer & Schulte, 1992) and it is expected that the use of labelled lectin probes in clinical diagnostics will increase.

Interaction with gut bacteria

Some lectins directly interact with resident bacteria of the digestive tract (Bainther *et al.*, 1993) and agglutinate them. These are then selectively removed from the lumen. Therefore, diets which contain these lectins can change the bacterial ecology of the gut (Pusztai *et al.*, 1990b). However, lectins can also interfere with the attachment of bacteria to the brush border by speeding up the turnover of epithelial cells and, therefore, changing the expression of receptor glycans on the gut surface (Pusztai, 1991). The best known example of this is PHA-induced small intestinal growth leading to the

proliferation of mannose-sensitive, type 1 fimbriated *Escherichia coli* in the rat gut (Wilson *et al.*, 1980; Ceri *et al.*, 1988; Banwell *et al.*, 1988). As shown recently (Pusztai *et al.*, 1993), the accelerated cell turnover induced by PHA acting as a growth signal leads to an increase in the proportion of juvenile cells on the small intestinal villi. These cells contain mainly polymannosylated membranes and cytoplasmic glycans in contrast to the fully differentiated, mature epithelial cells whose membranes contain complex glycosyl residues. This increase in the number of receptors with terminal mannose residues opens the way for more *E. coli* to be attached to the gut wall which can then successfully outcompete all other microorganisms resident in the gut.

Chemical probiosis

As the attachment of bacteria (Sharon, 1987) or parasitic protozoa (Lev *et al.*, 1986) to carbohydrate receptors on epithelial cells is mediated

mainly by lectin adhesins, it is possible to change the bacterial population of the small intestine by damaging its epithelium or by interfering with microbial adhesion. Thus, bacterial counts in the small intestine are increased over normal values if, by binding to different receptors on the same epithelial cells, the damage to their membranes by the food lectins and bacterial adhesins is additive or synergistic (Puzsai *et al.*, 1990b). In contrast, the administration of dietary saccharides and/or glycoconjugates, which structurally resemble the receptors of the gut wall, may reduce bacterial counts in the digestive tract. This process is selective and, by competitively inhibiting the attachment and consequent proliferation of a particular species, leads to the removal of bacteria whose lectin adhesin specifically interacts with the glycans used as probiotic agents. More significantly, food lectins which mimic the specificity of a particular bacterial adhesin have been used successfully to block the attachment to the mucosa of that species of bacteria. Accordingly, chemical probiosis is a process designed to reduce the population of harmful commensal bacteria, prevent infection by pathogens and/or promote the growth of beneficial microorganisms; this is achieved by the use of natural feed additives of lectins and/or glycoconjugates which block or compete with bacterial adhesins or modify the glycosylation of surface receptors for bacteria (Puzsai *et al.*, 1990b). Practical applications of chemical probiosis for the blockage of harmful bacterial proliferation in the small intestine of man and animals by dietary means are now well advanced (Fuller, 1989, 1992). Thus, high concentrations of dietary mannose supplements have been found to be effective in reducing salmonella infection in chicken (Oyoso *et al.*, 1989), with possibly beneficial consequences for humans. Indeed, most food/feed manufacturers market various probiotic products containing carbohydrate-based active ingredients. However, lectins are more powerful probiotic agents as their amounts required for blocking adhesion sites for bacteria are small compared with the high sugar concentrations needed for competitively inhibiting bacterial binding. For example, a daily dose of about 40 mg of *Galanthus nivalis* agglutinin, GNA, effectively stopped the PHA-induced coliform overgrowth in the rat small intestine (Puzsai *et*

al., 1993). In contrast, considerably more carbohydrate-based supplements were needed to achieve a significant reduction in *E. coli* counts. For example, the amount of glycoprotein (egg albumin) enriched for poly-mannosyl groups needed to significantly reduce *E. coli* counts in the rat was ten times that of GNA (Puzsai *et al.*, unpublished). The use of plant lectins for the blockage of pathogenic infections is now protected by an International Patent Application, No1 PCT/GB91/02236.

The role of endocytosis in lectin toxicity

Because lectins are endocytosed by gut epithelial cells or absorbed systemically only when bacteria are present in the digestive tract, lectins are toxic in animals with a conventional microflora but not in their absence (Rattray, Palmer & Puzsai, 1974; Jayne-Williams & Burgess, 1974). As apparently PHA is more toxic for humans living under poor nutritional and sanitary conditions (Griebel, 1950) or children (Haidvogel, Frisch & Grubbauer, 1979), this may be one of the reasons for the variable toxicity of PHA in the human diet (Rainer, 1962; Bender & Realdi, 1982).

Binding and endocytosis by the stomach and the large intestine

Although studies in model systems have indicated that the reaction between most lectins and their specific ligands is abolished at acid pH (pH 3 or less), lectins can apparently bind to the stomach epithelium *in vivo* even when the luminal pH is 3 or less (Hsu & Raine, 1982; Puzsai, 1991). This may, in part, explain why the presence of lectins in the diet slows down stomach-emptying in rats (Sgarbieri, Clarke & Puzsai, 1982).

After the villus tip cells, with the bound lectin still attached, are shed into the lumen of the small intestine, they are digested and their content recycled. However, due to their resistance to proteolytic breakdown, the released lectins can react with cells in more distal parts of the gut, the caecum and colon. Thus, PHA is not degraded by bacterial proteases and remains immunologically and functionally intact even in the feces (Puzsai, 1991).

Clearly, as most lectins survive the passage through the alimentary tract and bind to and are endocytosed by epithelial cells, they may serve as specific and site-directed targeting agents for

delivering drugs, micronutrients and minerals (Pusztai, 1989b). The design of lectin-conjugated delivery systems for medical applications is now being actively explored.

Effects of lectins on the gut immune system

Type 1, immediate hypersensitivity reactions by the gut immune system occur generally to even highly degradable food proteins. Allergic reactions to the more stable lectins which persistently bind to brush border cells are more extensive. For example, the anaphylactic response of the gut to PHA, as assessed from the leakage of ^{125}I -labelled serum proteins into the gut of rats given a single intragastric dose of lectin, is appreciable even on first exposure (Greer & Pusztai, 1985). This increased vascular permeability is not IgE-mediated but due to the direct degranulation of submucosal mast cells by PHA. This also occurs *in vitro* and is due to the cross-linking of membrane glycans by the multivalent PHA (Bach & Brashler, 1975). However, the allergic response was elevated in rats prefed on PHA diets for 11 days (Greer & Pusztai, 1985), suggesting that it was amplified by the formation of anti-PHA IgE.

Systemic effects

Lectins may influence systemic metabolism by two different but possibly simultaneous mechanisms (Pusztai, 1991). By reacting with neuro-endocrine cells of the gut they can indirectly influence the endocrine system of the body. Alternatively, by mimicking the effects of the endocrine hormones, the absorbed lectins may induce changes in the morphology and function of the endocrine organs and other peripheral tissues. Consequently, some lectins may directly interfere with metabolic pathways which are under the control of the endocrine system. The organs most often affected are the pancreas, skeletal muscle, liver, kidneys and thymus (Oliveira, Pusztai & Grant, 1988).

Oral immunization

Although most food proteins are rapidly degraded in the small intestine, nutritionally insignificant amounts are absorbed systemically through M cells of the gut-associated lymphoid tissue and presented by macrophages to competent lymphocytes of the immune system (Pusztai, 1989b). However, as allergic responses to

absorbed proteins in adults are minimized by T suppressor cells, few food proteins have harmful effects. In contrast, the luminal concentration of the more stable lectins is high and, consequently, their transport through the gut wall exceeds that of other antigens by several orders of magnitude (Pusztai, 1989b). Thus, in rats dosed with PHA up to 10% of the lectin is found in circulation 3 h after feeding (Pusztai, Greer & Grant, 1989). Therefore, PHA is a powerful oral immunogen and produces a high titre of monospecific anti-PHA antibody of IgG type in animals, including ruminants (Pusztai, 1989; 1991) and with some anecdotal evidence that the same happens in humans. However, not only PHA but also other lectins given orally are immunogenic (Pusztai *et al.*, 1981; Aizpurua & Russel-Jones, 1988). A recent demonstration of this was the finding of high titre anti-banana lectin (*BanLec-f*) IgG₁ antibodies in pooled human blood. Indeed, it was suggested that banana lectin might be used as a carrier for oral antihapten immunization in humans (Koshie *et al.*, 1992).

The time-course of antibody development shows the usual features of immunization and the primary response to PHA becomes measurable 10 days after the first dose. Further feeding or re-introduction of the lectin into the diet after PHA-free periods results in booster effects (Fig. 2). Thus, the putative gut anti-lectin-s-IgA system must be ineffective since it cannot prevent the absorption of PHA after its re-introduction (Pusztai, 1989a; 1991). This abrogation of gut s-IgA response to PHA and, possibly, to other lectins, may remove one of the major obstacles to the repeated administration of lectin-targeted oral drug conjugates.

Lectins can modulate IgE and IgG₁ responses to other antigens. For example, concanavalin A enhances the synthesis of anti-ovalbumin IgE in mouse (Gollapudy & Kind, 1975) and PHA induces changes in the IgE response to ovalbumin depending on the time of injection (Astorquiza & Sagayo, 1984). Furthermore, in DBA/2 mice orally sensitized to jacalin, a β -galactose-binding lectin from jackfruit (*Artocarpus heterophyllus*), and parenterally immunized with ovalbumin, IgE responses to both jacalin and ovalbumin were increased in a time- and dose-dependent way, suggesting that the lectin was a mitogenic immunomodulator for the production of IgE antibodies for both antigens, but that it

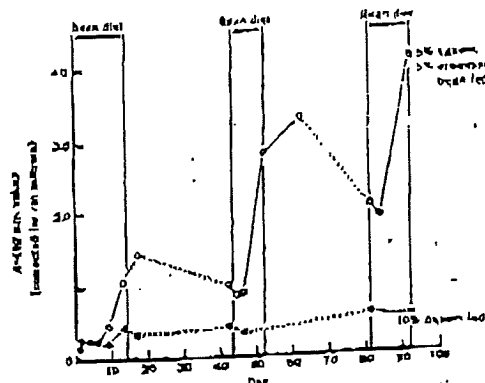


Fig. 2. The time-course of development of systemic anti-PHA, IgG-type antibodies in rats fed alternately for periods of 5 days on kidney bean proteins or control diets. Rats were blood-sampled throughout the experiment and the concentration of anti-PHA was measured by an ELISA assay. The reaction was visualized with 3,3'-diaminobenzidine after the anti-PHA from rat serum was reacted with peroxidase-labelled anti-rat IgG antibody.

had no effect on IgG, antibodies against the same antigens (Eastum-Miquel & Provuost-Danon, 1985). The use of lectins to reduce or abolish IgE responses to allergens, without affecting the synthesis of normal IgG-type humoral antibodies, holds out great promise for the possible clinical treatment of allergies in future.

Effects of dietary lectins on the pancreas

Most dietary lectins produce trophic changes within the exocrine pancreas which parallel the growth of the small intestine (Table 2). The best known example is PHA, which is growth factor for both the gut and pancreas. Through its effects on pancreatic secretion, PHA alters the hormone balance of the body (Pusztai, 1991; Pusztai *et al.*, 1992). SBA also induces pancreatic growth but may have only slight effects on blood insulin levels. The trophic effect of lectins on the pancreas is intimately involved with polyamine metabolism in the tissue (Table 2). However, the mechanism of lectin-induced, polyamine-dependent pancreatic growth appears to involve a route of hormonal mediation which is, at least in part, different from that caused by soya trypsin inhibitors and mediated solely by cholecystokinin (Pusztai *et al.*, 1992).

As secretion of digestive enzymes by pancreatic acini is severely reduced, for example, in

Table 2. Effect of lectins and trypsin inhibitor on the pancreas

	Weight	Acinar area	Polyamines
PHA	145	156	140
RPA; Robinia	143	n.d.	126
SNA-II; elder	136	150	140
SBA; soya	132	145	153
WGA; wheat germ	118	n.d.	114
DSA; Datura	111	n.d.	101
UDA; nettle	98	n.d.	94
SNA-I; elder	132	132	133
MAA; Maaekia	132	132	106
STI; trypsin inhibitor	135	151	160

The weight, acinar area of true transverse pancreatic sections (stained with haematoxylin and eosin, perimeter traced by computer-linked pixel planimetry and measured by a Joyce-Loebl 'magiscan' image analyser) and polyamine content of pancreas of rats fed with diets containing 0.7% lectins or trypsin inhibitor are expressed relative to those of the controls (taken as 100).

chronic pancreatitis, it is possible that the administration of controlled doses of non-toxic lectins which stimulate pancreatic hypertrophy may find use in clinical practice. Although stimulation of the pancreas by soyabean trypsin inhibitors has been tried in humans but with no great success (Liener *et al.*, 1988), it is possible that lectins may be more effective. This is because although trypsin inhibitors stimulate the secretion of proteases into the duodenum, these are partly inactivated by the trypsin inhibitors also present in the lumen. In contrast, lectins do not inhibit pancreatic proteases, and therefore most of the enzymes secreted remain active and contribute to the digestion of nutrients in the small intestine.

Effects on hormone balance

Most of the effects of lectins on systemic metabolism may be mediated through changes in circulating insulin levels. Thus, insulin concentration is depressed in rats fed on kidney bean diets or incubated with pure PHA, but the absence of major changes in blood glucose indicates that the animals are not diabetic (Pusztai *et al.*, 1991b). It is also clear that as a single acute oral dose of kidney bean albumins containing PHA leads to a significant but transient decrease in blood insulin (Fig. 3), its depression is not due to inadequate nutrition but

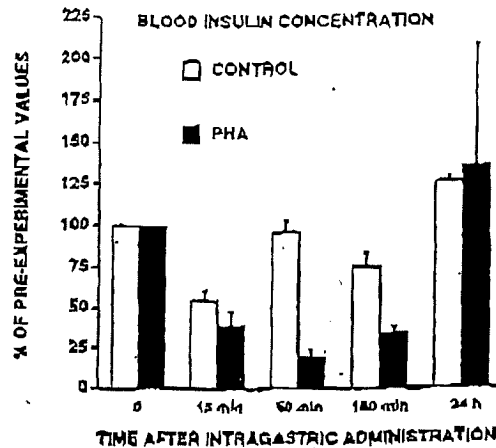


Fig. 3. Effect of the intragastric administration of a single dose of kidney bean lectin, PHA, on insulin concentration in blood circulation. Insulin was measured by an RIA assay on serum samples obtained from rats after intragastric intubation with a sample of 20 mg of kidney bean albumin (PHA concentration of about 7 mg). The results are expressed as percentages of 0-time values.

probably is a direct effect of the lectin on insulin synthesis and/or secretion by the pancreas.

To compensate for the depression of concentration of blood insulin caused by PHA and its metabolic consequences, various homeostatic processes are activated, including changes in other hormone levels. The parallel increase in glucagon concentrations (Grant *et al.*, 1987) or changes in levels of glucocorticoids confirm that dietary PHA does not only modulate insulin levels but can also induce complex changes in the hormone balance of the body.

It is generally believed that eating legume proteins is beneficial for human health because they appear to reduce the concentration of plasma and body lipids, although it is not clear whether lectins are involved in this process. However, PHA and, more importantly, other lectins with less antinutritional effects (Ng, Li & Yeung, 1989; Pusztai, 1991) do definitely have strong lipolytic effects. Despite the evidence that, due to a compensatory secretion of metabolic hormones and other homeostatic processes, the reduction in circulating blood insulin levels occurs without a corresponding increase in blood glucose, no clinical or medical uses for lipolytic lectins have been reported up till now.

Effects on peripheral organs and tissues

Skeletal muscle

Although the PHA-induced reduction in blood insulin is counteracted by compensatory changes in other hormone levels, this does not apply to all systemic effects of PHA. For example, PHA mimicks most of the *in vitro* biological effects of insulin (Pusztai & Watt, 1974) and even binds to the insulin receptor of muscle cells (Pusztai, 1991), but it does not elevate their protein synthesis rate. The most striking consequence of this is that rats lose about 30% of their skeletal muscle after receiving kidney bean diets for 10 days (Oliveira *et al.*, 1988). Although the mechanism of this atrophy is not fully clear, one of the main causes is the PHA-induced reduction in the fractional rate of protein synthesis in skeletal muscle; this occurs without a similar decrease in protein degradation rate, leading to a net loss of muscle protein (Palmer *et al.*, 1987). It is therefore possible that the atrophy results from abrogation of the stimulatory effect of insulin on muscle protein synthesis because the muscle receptor sites for insulin are blocked by PHA. However, the muscle wastage is not as extensive as could be expected from the low blood insulin concentration, because a parallel compensatory upregulation of the mRNAs coding for insulin-receptor and insulin-sensitive glucose transporter by the dietary PHA increases the efficiency of insulin-receptor interaction (Knot *et al.*, 1992). Nevertheless, even under these conditions, relatively large amounts of nutrients and polyamines are released from the atrophying skeletal muscle and are then used to support the growth of the PHA-stimulated gut (Bardocz *et al.*, 1992).

Thymus, spleen and other organs

PHA (Oliveira *et al.*, 1988) or other toxic lectins (Pusztai, 1991) induce the atrophy of the thymus and spleen. Some of these changes are irreversible and have potentially serious consequences for the immune system, especially T-cell-mediated immunity.

Similar to skeletal muscle, lectins may also affect the heart by reducing its weight and the rate of protein synthesis in it by the second day of feeding (Palmer *et al.*, 1987). Some lectins cause a slight reduction in liver weight by losing mainly lipids and glycogen after 10 days of feeding on diets containing kidney bean or

PHA (Grant *et al.*, 1987). The kidneys are slightly enlarged after feeding rats on diets containing antinutritive lectins for 10 days. However, this effect is almost certainly not specific as other forms of protein deprivation lead to similar enlargement. Although these effects of lectins on organs have been shown in animal studies, it is expected that some of them may also apply to humans.

Conclusion

Lectins are one of the most important physiologically active ingredients of our diet and provide potent exogenous biological signals. Although their amounts in the food vary greatly, lectins can have dramatic effects on the entire digestive tract and its bacterial population and on metabolism and health. Their extraordinary effectiveness stems from their resistance to

proteolysis, coupled with a high and specific chemical reactivity with endogenous surface receptors of the epithelial cells of the gut. Lectins are powerful oral and parenteral immunogens and some of their physiological effects are intricately linked to their interference with immune function. However, the primary effects and the potency of lectins as biological signals are a direct reflection of their specific chemical reactivity with saccharides. These reactions are predictable and the use of lectins in clinical-medical applications to improve metabolism and health of the gut and the body offers great promise.

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Lectin-induced increase in clonogenic growth of haematopoietic progenitor cells

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Abstract: The galactoside-specific plant lectin, *Viscum album* agglutinin (VAA-I) increases cellular parameters of natural host defence. It also binds to a variety of haematopoietic cells, including progenitors. We investigated whether VAA-I has a stimulatory effect on haematopoietic progenitor cells. Peripheral blood progenitor cells from 7 healthy volunteers were cultured in a colony assay with VAA-I plus erythropoietin (EPO) and stem cell factor (SCF). At 50 pg/ml VAA-I induced a significant increase in the cytokine-dependent clonogenic growth (52% in median, $p < 0.05$). In another set of experiments purified CD34⁺ cells were isolated from the bone marrow aspirate of 4 patients with non-metastatic breast cancer using fluorescence-activated cell sorting. Binding to CD34⁺ cells was demonstrated by using directly fluorescence-conjugated VAA-I. Co-incubation with D-galactose significantly abrogated this effect. CD34⁺ cells were cultured in the presence of EPO, SCF, interleukin-3, granulocyte/monocyte colony-stimulating factor and granulocyte colony-stimulating factor. VAA-I alone had no measurable effect on the clonogenic growth of the isolated cells. However, at concentrations of 100 and 250 pg/ml VAA-I increased the cytokine-dependent proliferation and differentiation of CD34⁺ cells by a median of 75 and 85%, respectively. The results show that VAA-I binds to haematopoietic progenitor cells and has a co-stimulatory effect on their proliferation.

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Viscum album agglutinin (VAA-I) belongs to the family of type II ribosome-inactivating proteins, such as ricin, abrin and modeccin. It has a molecular weight of 63 kDa (1, 2). Its application in small and non-toxic doses *in vivo* caused dose-dependent elevations in cellular parameters of the natural host defence (3). The ligand-binding capacity mediated by the B-chain is essential for the immunomodulatory effect of VAA-I. D-galactose inhibits the binding of the lectin to cellular galactose residues and its *in vitro* activity (4). In addition, the sugar-binding B-chain has been reported to be responsible for the lectin-induced gene expression and secretion of interleukin (IL)-1, IL-6 and tumour necrosis factor- α in cultures of human peripheral blood mononuclear cells (PBMC) (4, 5). VAA-I binds to various cells of the immune system: the highest affinity was observed on monocytes. VAA-I binding was also observed on CD34⁺ progenitor cells from normal bone marrow (6). Functional studies have not yet been reported. Other lectin-sugar interactions had

been discussed previously as one element in induction of proliferation and differentiation of haematopoietic progenitor cells (7).

We have investigated the effect of the galactoside-specific plant lectin VAA-I on circulating haematopoietic progenitors and CD34⁺ cells from bone marrow aspirates.

Material and methods

Isolation of VAA-I

VAA-I was isolated according to previously published methods (5, 8). Briefly, homogenates from the leaves of *Viscum album* plants were ultrafiltrated using 2 filtration modules: cellulose triacetate with 20 kDa cut-off and polysulfon with 100 kDa cut-off, respectively. The product was purified further on lactose-agarose columns (Sigma-Aldrich Handels GmbH, Vienna, Austria) as follows: the ultrafiltrate, diluted with a buffered

salt solution (PiCM), was recirculated overnight with a flow rate of 1.5 ml/h; 0.1 M galactose- or lactose-containing buffer was used to elute high-affinity bound VAA-I (9). The pure VAA-I was dialysed against the PiCM buffer and concentrated to 0.8 mg/ml. The sugar-binding lectin content was measured by an enzyme-linked lectin assay as described previously (3). Endotoxin contamination was measured by Dr K.B. Becker with a quantitative kinetic limulus amoebocyte-lysate assay (10).

The purified VAA-I was conjugated with FITC by carbonate buffer pH 9.0, and the labelled lectin separated on a Sephadex G-15 column.

Healthy volunteers and patients

Peripheral blood progenitor cells (PBPC) were obtained from 7 healthy donors. All volunteers gave informed consent. Bone marrow (BM) aspirates were obtained from patients with non-metastatic breast cancer as a part of the staging procedure.

Isolation of PBPC and CD34⁺ cells from BM

Peripheral blood (PB) or BM samples were collected in heparinized blood collection tubes. Samples were diluted 1:2 in Hanks' balanced salt solution (Gibco, Scotland, UK) and layered over Lymphoprep (Nycomed AS, Norway). After centrifugation at 800 g for 20 min cells of the interphase were collected and washed twice at 300 g for 10 min. A final washing step was performed at 200 g in order to reduce platelet contamination. Cell counts were determined in a haemocytometer using acridine orange for vital staining.

BM mononuclear cells were incubated for 30 min with FITC- or PE-conjugated anti-CD34 antibody (HPCA-2, Becton-Dickinson, San Jose, CA, USA). Isolation of CD34⁺ cells was performed on a fluorescence-activated cell sorter (FACS Vantage, Becton-Dickinson) equipped with a 488 nm laser using phosphate buffered saline (PBS) as sheath fluid. Haematopoietic progenitors were identified by expression of CD34 and by light scatter profile in the blast gate. Sort purity was assessed on a FACScan (Becton-Dickinson) and was $\geq 96\%$. No significant monocyte contamination was detected by CD14 staining.

VAA-I binding assay

CD34⁺ cells of BM (1×10^5 /well) in Dulbecco's modified PBS containing 0.02% NaN₃ and 0.5% bovine serum albumin were stained directly with FITC-VAA-I and PE-conjugated anti CD34 according to standard staining procedures. As controls both a 100-fold concentration of unlabelled

VAA-I and an isotope control (PE-mouse IgG) were used. In order to investigate the inhibitory effect of sugar on binding of VAA-I to CD34⁺ cells simultaneously 20 and 50 mM D-galactose, respectively (Merck, Switzerland), was given. For each test 10^4 cells were analysed on a FACScan equipped with Lysis II software. The gating of cells was defined by a combination of FSC and SSC cytometric parameters. Cell viability, as tested by trypan blue exclusion assay, was 98% (± 1.5).

Haematopoietic colony-forming assay

The colony-forming capacity of stem cells was investigated in methyl cellulose cultures. Cells were mixed with methyl cellulose medium consisting of 0.9% methyl cellulose (4000 centipoises; Sigma, USA), 0.6% bovine serum albumin (Boehringer, Germany) and 30% pretested fetal calf serum (Gibco, Germany) in Iscove's modified Dulbecco's Medium (Gibco, Germany) containing 50 μ M 2-mercaptoethanol and 2 mM L-glutamine (Gibco, Germany). Final concentrations were 1.5×10^5 /ml for PBMC and 1×10^3 /ml for purified BM cells. Cultures were plated in 35 mm Petri dishes (Greiner, Germany) in the presence of growth factors: 50 ng/ml G-CSF (granulocyte colony-stimulating factor) (Amgen/Roche, Germany), IL-3 and GM-CSF (granulocyte/macrophage colony-stimulating factor) (Sandoz, Germany), 1 U EPO (erythropoietin)/ml (Boehringer, Germany) and 20 ng SCF (stem cell factor)/ml (Genzyme, Germany). The concentration of each growth factor was chosen well into the plateau phase of the appropriate dose-response curves (data not shown). The plates were incubated at 37°C under a fully humidified atmosphere and 8% CO₂. Colonies were scored after 10–14 d under a dissection microscope (Zeiss, Germany). Cell aggregates containing more than 50 cells were scored as colonies. Morphological analysis was performed on cytocentrifuge preparations of single colonies after Pappenheim staining.

Statistical analysis

Wilcoxon's and Student's *t*-test were used to analyse the lectin-induced responses in numbers of cell colonies using the Statgraphics statistical package for IBM-compatible computer.

Results

In vitro effect of purified VAA-I on PBPC

The effect of VAA-I on cytokine-induced proliferation of PBPC was studied in 7 healthy volunteers. PBPC were incubated with purified VAA-I at

various concentrations between 25 pg/ml and 1 ng/ml, 1 U EPO/ml and 20 ng SCF/ml. VAA-I alone without the addition of growth factors was ineffective in influencing the clonogenic proliferation of PBPC. VAA-I, however, increased the number of growth factor-induced colonies. The maximum enhancement detected at 50 pg VAA-I/ml was 52% in median ($p < 0.05$) resulting in a stimulation index of $2.1 (\pm 0.39)$ at this concentration (Table 1). Morphological investigation of single colonies from 4 experiments revealed that the growth of erythroid and granulocyte/macrophage progenitor cells was stimulated. Mean increase was $1.8 (\pm 0.6)$ for erythroid colonies ($p = 0.09$) and $1.8 (\pm 0.29)$ for neutrophil colonies ($p = 0.03$) (Table 1).

Binding of VAA-I to CD34⁺ BM cells

In order to obtain more insight into mechanisms underlying the colony growth-enhancing effect of VAA-I on CD34⁺ cells the binding of FITC-VAA-I to sorted CD34⁺ BM cells was tested using a flow cytometric analysis. In duplicates from 2 donors the mean percentage of positively stained CD34⁺ cells (\pm SEM) was $83.5 \pm 1.5\%$. Figure 1 shows a representative example of this investigation. D-galactose as an inhibitor of lectin-binding to cellular galactoside residues caused a significant decrease in the percentage of positively stained cells ($p < 0.05$).

Effect of VAA-I on CD34⁺ BM cells

CD34⁺ cells were isolated from 4 bone marrow aspirates using fluorescence-activated cell sorting. BM samples were obtained from patients with non-metastatic breast cancer and normal haematopoiesis. VAA-I by itself showed no effect on the BM haematopoietic precursors. However, in combination with EPO, SCF, G-CSF, GM-CSF and IL-3, VAA-I had a stimulatory effect on the CD34⁺ BM cells with a dose-dependent relationship. The maximum colony increase occurred at 250 pg VAA-I/ml, but 50 pg and more markedly 100 pg VAA-I/ml were also able to induce an elevation in the colony number. Median values of colony numbers were $10.2 (\pm 5.4)$ in the absence of VAA-I, $21.0 (\pm 3.3)$ and $24.5 (\pm 6.1)$ in the presence of 100 pg or 250 pg lectin/ml, respectively (Fig. 2). Twenty mM D-galactose was able to abrogate these effects (data not shown).

Discussion

Regulation of proliferation and differentiation in haematopoiesis requires a complex interactive network of positive and negative stimulatory signals. Within the past 15 yr research has identified several

Table 1. *In vitro* effect of VAA-I on clonogenic growth of PBMC in the presence of 1 U EPO/ml and 20 ng SCF/ml. Stimulation index of the number of colonies (SI = experimental value/control value) after 12 d incubation of 1.5×10^5 PBMC/ml from healthy volunteers is shown

	VAA-I (pg/ml)				
	0	25	50	250	1000
SI of total number of colonies					
Average	1.0	1.0	2.1*	1.7	1.0
SEM		0.1	0.4	0.4	0.1
SI of erythroid colonies					
Average	1.0	1.0	1.8	1.3	1.0
SEM		0.1	0.6	0.3	0.3
SI of neutrophil colonies					
Average	1.0	1.0	1.8*	1.1	1.05
SEM		0.1	0.3	0.4	0.2

* $p < 0.05$

soluble glycoproteins including SCF, IL-1, IL-6 and flt3 ligand, which efficiently stimulate the growth of primitive progenitor cells. These are immunophenotypically characterized by expression of CD34 and functionally identified by the ability to form diverse lineage-specific colonies. Another group of substances with potential influence on haematopoiesis are lectins. These have previously been shown to stimulate the function of immunocompetent cells. Previous analyses showed that the galactoside-specific lectin VAA-I binds not only to lymphocytes and monocytes with high affinity but also to haematopoietic progenitors.

Formerly, binding of VAA-I to CD34⁺ cells was demonstrated by using directly fluorescence-conjugated material. Specificity was shown by its almost complete abrogation through blocking with D-galactose. This assay had been used previously for demonstration of lectin binding to multiple haematopoietic subpopulations (5). It has been reported recently that lectins may further subdivide CD34⁺ progenitors in functionally defined subgroups (7). The plant lectin *Ulex europaeus* binds to about half of the CD34⁺ cells. Sorting and functional analysis of these cells in colony assays revealed that this subpopulation was highly enriched for erythroid progenitors (BFU-E).

We initiated a study of the potential functional effect of VAA-I on CD34⁺ cells. It has been shown previously that a small number of pluripotent progenitor cells circulates in the PB of healthy individuals. Our first set of experiments showed that the colony formation of PBPC was not influenced by the addition of VAA-I as a single agent to the colony assay. However, it significantly enhanced the clonogenic potential of SCF+EPO-induced colony formation. Since monocytes have

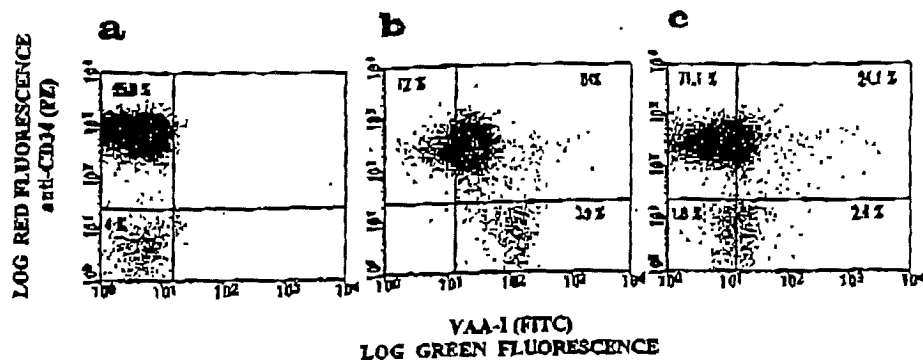


Fig. 1. A representative set of dot plots showing that addition of D-galactose blocks the binding of VAA-I. Separated BM cells were stained with unlabelled VAA-I and PE-anti CD34 (a), with FITC-VAA-I and PE-anti CD34 (b), and FITC-VAA-I and PE-anti CD34 after the addition of 50 mM D-galactose (c).

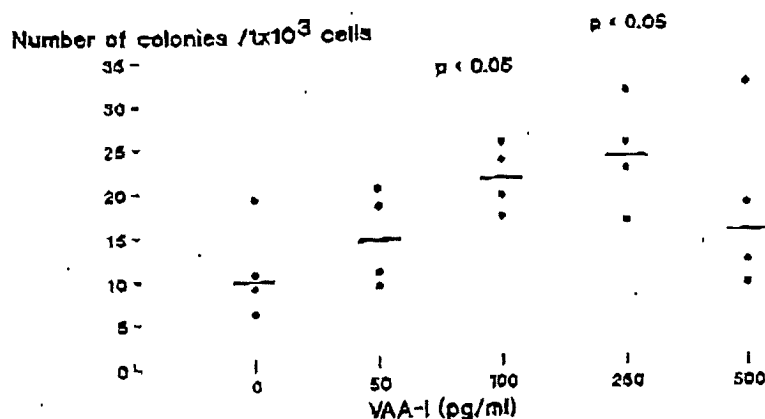


Fig. 2. Effect of VAA-I on CD34⁺ BM cells in the presence of 1 U EPO/ml, 20 ng SCF/ml, 50 ng G-CSF/ml, 50 ng GM-CSF/ml and 50 ng IL-3/ml. Number of colonies after 10–14 d of 1×10^3 CD34⁺ cells/ml from BM of 4 patients with cancer were shown as points, the bars represent the median value for each group.

previously been identified as a prime target of VAA-I and are potent secretors of soluble cytokines, we performed a second set of experiments in order to reduce a potential, monocyte-mediated proliferative effect. CD34⁺ progenitors were highly purified by fluorescence-activated cell sorting using bone marrow aspirates from patients without haematopoietic dysfunctions. Contamination by significant populations of monocytes or lymphocytes was excluded by gate setting and quality control of sorted cells. VAA-I increased the cytokine-induced proliferation of CD34⁺ cells inducing erythroid and granulocyte-macrophage colonies. The abrogation of the stimulatory effect of VAA-I by D-galactose indicates the importance of lectin-sugar interactions in this process. Moreover, VAA-I was ineffective as a single agent.

The exact mechanism of the VAA-I effect is not yet clear. Interaction of cytokines for optimal stimulation of haematopoietic progenitor growth is a common phenomenon, which may be mediated

through upregulation of receptor numbers, influence of receptor affinity, facilitation of signal transduction or suppression of growth inhibitory signals. Since our assay system included cytokines and VAA-I during the whole incubation period, different interaction pathways cannot be elucidated. The VAA-I binding to CD34⁺ progenitor cells underlines a direct stimulatory effect of CD34⁺ cells. In comparison to the above-mentioned results on the *Ulex europaeus* lectin, VAA-I does not seem to have a limited effect on a specific functionally defined subset of progenitors. Stimulation occurred in fully haemoglobinized erythroid and myeloid colonies with no apparent preference. These experiments point to a potential general enhancement of cytokine-mediated stimulatory signals, rather than to a lineage specific function.

This synergistic effect of VAA-I opens up a new perspective within lectin research and may reveal a new role in the *in vivo* application of these substances.

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GLYCOSYLATION SITE OF THE MAJOR ALLERGEN FROM
OLIVE TREE POLLEN. ALLERGENIC IMPLICATIONS OF
THE CARBOHYDRATE MOIETY*

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Abstract—The electrophoretic analysis of purified *Ole e* 1, the major allergen from *Olea europaea* pollen, reveals the presence of two main variants, glycosylated (20.0 kDa) and non-glycosylated (18.5 kDa) components. The glycosylated variant has been identified as a concanavalin A-binding (18.5 kDa) components. The glycosylated variant has a molecular mass of about 1.3 kDa (5% weight of the glycoprotein). Its carbohydrate moiety has a molecular mass of about 1.3 kDa (5% weight of the glycoprotein). Its carbohydrate moiety has a molecular mass of about 1.3 kDa (5% weight of the glycoprotein). Enzymatic treatment of native *Ole e* 1 with the specific glycosidase PNGase F accounts for an oligosaccharide N-linked to the polypeptide chain. This treatment does not sensibly modify the secondary structure of the protein but diminishes the affinity of the allergen for specific IgE antibodies. Tryptic digestion of *Ole e* 1 reveals the presence of a single carbohydrate-containing peptide. This peptide was recognized by the sera of hypersensitive individuals. The amino acid sequence of this peptide is Phe-Lys-Leu-Asn-Thr-Val-Asn-Gly-Thr-Thr-Arg, asparagine at the seventh being the carbohydrate attaching site. The obtained data are discussed in terms of the potential role of the sugar moiety in the allergenic activity of *Ole e* 1.

INTRODUCTION

Allergic diseases are increasing in developed countries. More than 10% of the population is hypersensitive (Type I allergy) against natural or synthetic materials, which can originate severe symptoms including anaphylactic shock. Plant pollens are one of the most typical causes of allergy and usually exhibit seasonal character. Among hypersensitivities to tree pollens, allergy to the olive tree pollen is one of the most common in the Mediterranean area (Bousquet *et al.*, 1985; Wheeler, 1992).

Ole e 1, the major allergen from olive trees (*Olea europaea*) pollen, has been recently isolated (Villalba *et al.*, 1990). This allergen shows a characteristic electrophoretic pattern in SDS-PAGE, in which two main bands of different molecular mass (18.5 and 20 kDa) can be observed. This heterogeneity does not seem to be correlated with differences on primary structure since both forms had identical NH₂-terminal sequence, and was attributed to the existence of a glycosyl portion in the higher molecular weight form (Lauzurica *et al.*, 1988; Villalba *et al.*, 1990; Lombardero *et al.*, 1992).

Allergens from several biological sources have been characterized as glycoproteins (Ford and Baldo, 1986; Klapper *et al.*, 1980; Polo *et al.*, 1991; Heymann *et al.*, 1986; Weber *et al.*, 1987; Nilsen *et al.*, 1991; Chua *et al.*, 1988; Sánchez-Monge *et al.*, 1992). The elucidation of both the glycosylation site and the carbohydrate structure are often essential studies that must be performed in order to clarify the particular function of this moiety in the allergenic glycoproteins. However, the low recovery in the purification of most allergens and the relatively low sensitivity of the current methods for detecting the glycosylated forms have made this research difficult. So far, the determination of the whole sequence, from either the mature protein or the corresponding nucleotide segment, is frequently required to determine the putative glycosylation site of a protein (Griffith *et al.*, 1991). Methods recently described for detecting glycopeptides at the subnanomol level allow the identification of the carbohydrate attachment site of proteins isolated in low amounts, and the study of the role of this group in the molecule. This work deals with the identification of the glycosylation site in the olive tree pollen allergen. This report also initiates the study of the role of the carbohydrate in the allergenicity of the protein.

MATERIALS AND METHODS

Purification of Oleic I and related analytical procedures

Olea europaea pollen was purchased from Allergon AB. The preparation of the whole pollen extract and the purification of Ole e I were performed as previously described (Villalba *et al.*, 1990). The protein concentration of the purified allergen was determined by amino

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Abbreviations: CD, circular dichroism; Con A, concanavalin A; HPLC, high-performance liquid chromatography; HRPO, horseradish peroxidase; PNGase F, Peptide N-(acetyl- β -glucosaminyl)-asparagine amidase F; TBS, 50 mM Tris-HCl buffer, 0.5 M NaCl, pH 7.5; TFMS, trifluoromethanesulfonic acid.

acid analysis and spectrophotometric measurements by considering an extinction coefficient (0.1% w/v, 1 cm, 280 nm) of 0.90. Protein and peptides (1–5 nmol) were hydrolysed with 0.2 ml of 5.7 N HCl containing 0.1% (w/v) phenol, at 105°C for 24 hr, in evacuated and sealed tubes. Hydrolysates were analysed on a Beckman system 6300 amino acid analyser with an analog interface module of System Gold. SDS-PAGE was performed according to Laemmli (1970) in 15% acrylamide gels. The protein bands were detected by staining with Coomassie Blue. The relative amount of different protein components was estimated by gel scanning on a Beckman DU-8 spectrophotometer equipped with the corresponding accessory. Protein bands were electrophoretically transferred to nitrocellulose sheets on a LKB Novablot 2117 Electrophoretic Transfer Unit (LKB) at 1 mA/cm² for 1 hr, according to Towbin *et al.* (1979).

Carbohydrate analysis

Glycoproteins and glycopeptides were detected on nitrocellulose membranes (Hsi *et al.*, 1991) after either electrophoretic transfer from polyacrylamide gels or direct application. The nitrocellulose membrane was soaked in a blocking solution (TBS/2% polyvinylpyrrolidone) for 1 hr and then incubated with biotinylated-Con A lectin (10 µg/ml) in TBS/2% polyvinylpyrrolidone for 1 hr. Afterwards, the membrane was washed with TBS/0.1% Triton X-100 and incubated with Vectastain ABC reagent (avidine, biotinylated-HRPO) (Vector Laboratories) 1:400 diluted in TBS/1% polyvinylpyrrolidone for 30 min. Bound lectins were visualized by the HRPO reaction with 0.05% (p/v) 3,3'-diaminobenzidine-HCl, 0.03% (v/v) H₂O₂ in TBS for 1 min.

The evaluation of the carbohydrate component was performed by two different photometric assays: acid hydrolysis and colorimetric reaction with 2,2'-bicinchoninic acid (Waffenschmidt and Jaenicke, 1987), and periodic acid treatment and reaction with basic fuchsin (Mantle and Allen, 1978). D-Glucose and ovalbumin were used respectively as standard for these analyses.

Mass spectrometry

Time-of-flight plasma desorption mass spectrometry was performed on a Kompact Maldi III laser spectrometer. A pulse width of 3 ns and 23 shots were used. The spectrum was recorded at an acceleration voltage of 20 kV. The data were obtained in a Sun SPARstation for processing. Insulin (5737 Da), cytochrome c (12,387 Da) and cytochrome c dimer (24,774 Da) were used as molecular mass markers. The molecular mass of the carbohydrate moiety of the allergen was calculated from the difference between the experimental values obtained for the glycosylated and the non-glycosylated forms.

Chemical and enzymatic deglycosylation of Ole e I

Trifluoromethanesulfonic acid (TFMS) treatment of Ole e I (1 mg) was carried out for 2 hr at 0°C (Sojar

and Bahl, 1987). Enzymatic deglycosylation of the allergen was performed with PNGase F (EC 3.2.2.18, Boehringer Mannheim). Ole e I (1 mg) was incubated with PNGase F (20 units) for 18 hr at 37°C, in 0.5 ml of 100 mM Tris-HCl, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride and 1% (w/v) *n*-octylglucoside. Deglycosylated protein was purified on a Sephadex G-25 superfine column (1 × 28 cm) equilibrated in 0.2 M ammonium bicarbonate, pH 8.0.

Immunological assays

A pool of hypersensitive human serum samples, obtained from individuals selected from a larger population, which had the highest radioallergosorbent test (RAST 3–4) against olive tree pollen, was kindly provided by Dr C. Lahoz (Fundación Jiménez Díaz, Madrid).

ELISA inhibition assays were performed as previously described (Núñez-Arias *et al.*, 1990) with minor modifications. Microtiter plates were coated with 0.1 ml per well of native Ole e I (1 µg/ml) in PBS, and blocked with 3% normal human serum in PBS. Then 100 µl aliquots of olive pollen hypersensitive human serum (1:8 dilution), co-incubated with different concentrations of the inhibitor, were added and incubated for 4 hr at 37°C. Binding of the human IgE was detected by HRPO-labeled goat anti-human IgE (Nordic Immunology) at a 1:1000 dilution in PBS/1% normal human serum/0.05% Tween 20 and substrate.

For immunodetection, the nitrocellulose sheets with the transferred or dot blotted samples were equilibrated in PBS/0.1% Tween 20 for 15 min. After blocking with 3% (w/v) skim milk in PBS/0.1% Tween 20 for 2 hr, membranes were incubated with the hypersensitive human serum (1:8 dilution) for 20 hr. After washing, HRPO-labeled goat anti-human IgE was added at 1:1000 dilution, in PBS/1% skim milk/0.05% Tween 20, and incubated for 1 hr. The peroxidase reaction was developed in PBS as described above, except in the case of the dot blotted samples, which were visualized with ECL Western blotting reagent (Amersham).

Spectroscopic analyses

Ultraviolet-absorbance measurements were carried out on a Beckman DU-7 spectrophotometer at 5 nm/s scanning speed in 1 cm optical-path cells.

Circular dichroism (CD) spectra were obtained on a Jobin Ivon Mark III dichrograph, fitted with a 250 W xenon lamp. Samples were analysed in 0.05 cm optical-path cells, at a protein concentration of 0.1–0.5 mg/ml. The spectra were recorded in the far ultraviolet region, at 0.2 nm/s scanning speed. Mean residue weight ellipticities were calculated based on 111 as the average molecular weight per residue, obtained from the amino acid composition, and expressed in terms of $[\theta]$ (degree × cm² × dmol⁻¹). The reported CD values were the average of five independent determinations. Secondary structure estimations were performed by computer analysis of the CD spectra according to the ellipticity values of Bolotina *et al.* (1980).

All the samples were prepared in Milli-Q filtered water and centrifuged on a Beckman microfuge prior to being used.

Proteolytic treatment and sequence determination

Trypsin digestion of the reduced and carboxyamidomethylated *Ole e I* was performed as previously described (Menéndez-Arias *et al.*, 1988). Radioalkylated (iodo- ^{14}C)acetamide) allergen (30 nmol) was incubated with trypsin at 1/100 weight enzyme/substrate ratio, in 0.2 M ammonium bicarbonate for 2 hr, at 37°C. No insoluble material was detected after this treatment. The digested sample was chromatographed on a HPLC system equipped with a Nucleosyl C-18 reverse-phase column by using a gradient of acetonitrile in 0.1% trifluoroacetic acid. The eluent was continuously monitored at both 214 and 280 nm wavelengths. An aliquot (1%) of each collected fraction was analysed for both presence of carbohydrates and IgE recognition by the procedures above described.

Automatic Edman degradation of the single glycopeptide (0.8–1.0 nmol) isolated from *Ole e I* was performed on an Applied Biosystems model 477A sequencer. The resulting phenylthiohydantoin-amino acid derivatives were identified by using a model 120A on-line phenylthiohydantoin-analyser and the standard Applied Biosystems program (Hewick *et al.*, 1981).

RESULTS

Determination of the molecular mass of the carbohydrate moiety of *Ole e I*

The SDS-PAGE pattern of *Ole e I* shows the four bands (18.5, 20.0, 22.0 and 40.0 kDa) described by Wheeler *et al.* (1990) as the reactive forms of the major olive pollen allergen (Fig. 1a). The components of 20, 22 and 40 kDa contain carbohydrate groups since they are stained for sugars by the Con A lectin method after their transfer to nitrocellulose membranes (Fig. 1d). The bands of 18.5 and 20 kDa are the main components of the allergen since the other two forms (22 and 40 kDa)

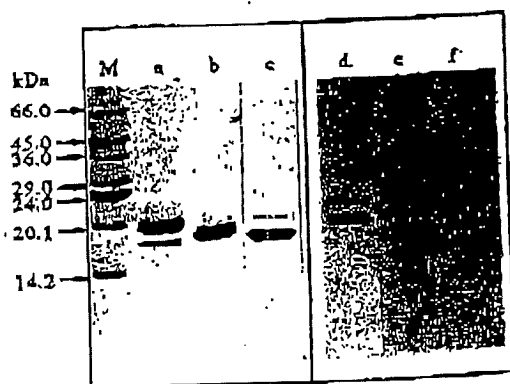


Fig. 1. SDS-PAGE analysis of (a, d) native, (b, e) TFMS-treated and (c, f) PNGase F-treated *Ole e I*: (a, b, c) Coomassie Brilliant Blue staining and (d, e, f) biotinylated-Con A staining. Five μg of protein was applied in each lane. M, molecular mass protein markers (BioRad).

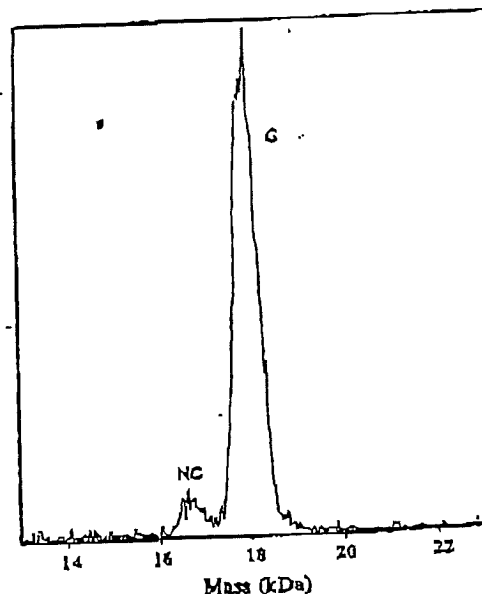


Fig. 2. Mass spectrometry analysis of the olive pollen allergen. The sample (20 μg of protein) was dissolved in 0.1% trifluoroacetic acid and dried over a multisample slide. G, glycosylated and NG, non-glycosylated forms of the allergen.

represent less than 5% of the total protein, based on the results from the densitometric scan of the gels. Among the two main bands, the relative amount of the glycosylated one (20 kDa) is 80–85% of the total allergen (Fig. 1a).

The carbohydrate component of *Ole e I* represents 3.5–4.0% of the total mass of the protein, according to the photometric assays. This value stands for 5% (w/w) of the main glycosylated variant (20.0 kDa), which would correspond to a molecular mass of about 1.0 kDa for the carbohydrate moiety. Mass spectrometry analysis of the olive pollen allergen gives a molecular mass of 17.9 ± 0.1 and 16.6 ± 0.1 kDa respectively for the glycosylated and non-glycosylated forms (Fig. 2). Therefore a molecular mass of 1.3 kDa is deduced for the carbohydrate component of *Ole e I* by this method.

Chemical treatment of native *Ole e I* with TFMS results in extensive deglycosylation of the molecule, as shown by SDS-PAGE and biotinylated Con A staining (Fig. 1b, e). The absence of stained bands in the presence of the biotinylated lectin attests the efficiency of the TFMS treatment. Moreover, no carbohydrate content is detected by photometric assay of *Ole e I* after this deglycosylation treatment. TFMS unspecifically removes oligosaccharide molecules N- and O-linked to glycoproteins, without any proteolytic effect. The apparent molecular mass of *Ole e I* treated with this reagent is coincident with that of the non-glycosylated form (18.5 kDa). A weak band, which does not stain for sugars, also appears at 19.5–20.0 kDa after this treatment, and it can be attributed to the deglycosylated form of the original 22.0 kDa band.

The native pollen allergen was also treated with N-glycosidase F (PNGase F), which specifically removes oligosaccharides N-linked to polypeptide chains. *Ole e I*

is completely deglycosylated during this enzymatic treatment as deduced from Fig. 1, since sugar-stained bands do not appear on the SDS-PAGE of the treated allergen. This result indicates that only N-linked carbohydrates are present in *Ole e 1*. A weak band also appears at 19.5–20.0 kDa, as occurs after the chemical treatment.

Secondary structure of deglycosylated *Ole e 1*

The circular dichroism spectra in the far-ultraviolet region (200–250 nm) of the TFMS- and PNGase F-treated *Ole e 1* have been recorded, and compared with that of the native protein (Fig. 3). Both the enzymatically deglycosylated and the native allergen exhibit identical spectra. This indicates that the secondary structure of the protein is maintained after this deglycosylation treatment. Secondary structure analysis of the allergen renders 12.9% α -helix, 29.1% β -structure, 22.7% β -turns and 35.3% aperiodic structure, expressed as percentages of the overall residues in the polypeptide chain. However, TFMS treatment of the allergen greatly changes the protein conformation since the periodically ordered secondary structure almost disappears (Fig. 3).

IgE recognition

SDS-PAGE, electroblotting and immunostaining experiments were carried out in order to assess the recognition of the deglycosylated *Ole e 1* by the IgE present in human sera from olive pollen hypersensitive individuals. As Fig. 4a shows, the four Coomassie-stained bands of native *Ole e 1* specifically bind human IgE. However, the TFMS-treated *Ole e 1* is not recognized by these IgE antibodies (Fig. 4b). This latter result is in agreement with that obtained from the ELISA inhibition experiment, where no complete inhibition

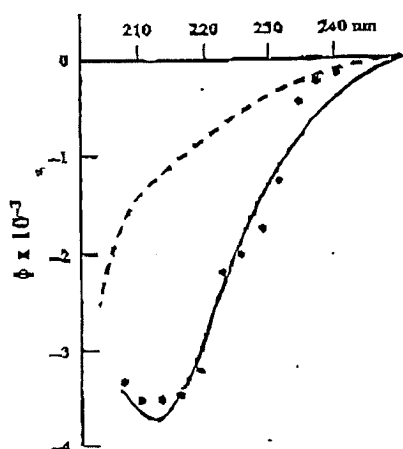


Fig. 3. Far-UV circular dichroism spectra of (—) native and PNGase F-treated *Ole e 1* and (---) TFMS-treated *Ole e 1*. The spectra were recorded in PBS, pH 7.2, at 25°C. A concentration of 0.3 mg/ml of protein was used. Circular dichroism values are expressed in terms of θ , mean residue weight ellipticity, in units of degree \times cm² \times dmol⁻¹. (●) CD theoretical values deduced from the secondary structure determined according to the reference parameters of Bolotina *et al.* (1980).

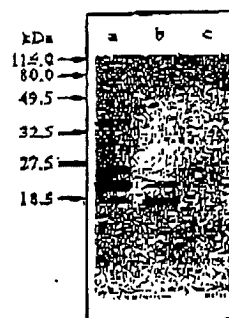


Fig. 4. Immunoblotting of (a) native, (b) PNGase F- and (c) TFMS-treated *Ole e 1*. Olive pollen hypersensitive human serum and HRPO-labeled goat anti-human IgE were used. Two μ g of protein were applied in each lane.

of native *Ole e 1*-IgE interaction is achieved even at high concentration of the TFMS-treated allergen (Fig. 5).

PNGase F-treated *Ole e 1* retains antigenic determinants since it is recognized by hypersensitive human sera after electrophoresis, transfer and immunostaining (Fig. 4b). However, the response of this deglycosylated allergen to the IgE antibodies is decreased in comparison to that of the native allergen. In fact, 10-fold higher concentrations of enzymatically deglycosylated protein than that of native allergen are required to obtain 50% inhibition in the ELISA competition assay.

Amino acid sequence at the glycosylation site

HPLC of the tryptic digestion of *Ole e 1* allows the separation of 20 peptides. Aliquots (1%) of each isolated peptide were tested for sugar recognition with biotinylated-Con A. Only one peptide gives a positive reaction (Fig. 6). The amino acid composition of this peptide is: Asx₁, Thr₃, Gly, Val, Leu, Phe, Lys, Arg. This peptide was sequenced by automated Edman

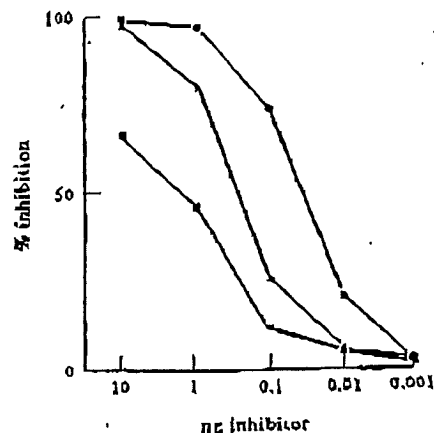


Fig. 5. Inhibition of IgE binding to *Ole e 1*-coated wells by (●) native, (■) TFMS-treated, and (▲) PNGase F-treated *Ole e 1*. Inhibitors were added after co-incubation with the human serum, and binding was determined after addition of HRPO-labeled goat anti-human IgE. Standard deviations were under 5% for triplicate samples in all the assays performed.

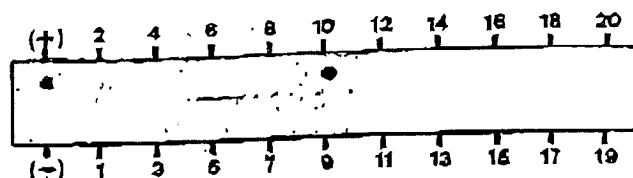


Fig. 6. Screening for sugar content of the purified tryptic peptides (from 1–20) obtained from *Ole e I*. HPLC isolated peptides (10–20 pmol each) were spotted on nitrocellulose membrane strips and stained with biotinylated-Con A lectin. Native *Ole e I* (+) and hen egg white lysozyme (–) were used as positive and negative controls respectively.

degradation and the primary structure found was FKLVNTV(-)GTTR. A gap appears at the seventh position during the Edman degradation, which would correspond to an Asx residue based on the above amino acid composition. Moreover, Thr was observed at the ninth position. It is well known that Asn-X-Ser/Thr is a consensus sequence for the attaching site of carbohydrates N-linked to polypeptide chains. Therefore Asn would be at the seventh position of the peptide. Thus, the proposed amino acid sequence at the glycosylation site in the olive pollen allergen is FKLVNTVNGTTR.

Finally, this tryptic peptide was directly applied to a nitrocellulose membrane and immunostained with a pool of olive pollen sensitive human sera. The positive reaction observed (Fig. 7) suggests the existence of an immunodominant epitope in this segment of the allergen.

DISCUSSION

The involvement of the glycosyl moiety of proteins in multiple biological functions of glycoproteins, i.e. the intracellular transport, the turnover rate or the protection against proteases, as well as its contribution to the conformation and antigenicity of the molecule is well known (Rademacher *et al.*, 1988). However, the knowledge about the particular role of the carbohydrate component in the allergenicity of glycoproteins is scarce, since only a few molecules have been studied from this point of view. Preliminary analyses about the properties of the oligosaccharide portion of several glycoallergens have demonstrated allergenic implications for this group (Nilsen and Smestad-Puilsen, 1986; Weber *et al.*, 1987; Sward-Nordmi *et al.*, 1988; Duffort *et al.*, 1991). The major allergen from olive tree pollen was suggested to be a glycoprotein (Lauzurica *et al.*, 1988; Villalba *et al.*,

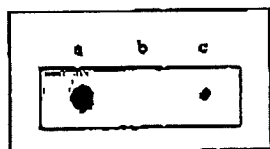


Fig. 7. Recognition of the tryptic glycopeptide from *Ole e I* (peptide 10 in Fig. 6) by hypersensitive human serum. Dot blots of (a) the tryptic glycopeptide (0.3 µg), (b) a non-glycosylated tryptic peptide (peptide 20 in Fig. 6) (0.5 µg) as negative control, and (c) native *Ole e I* (0.2 µg) as positive control.

1990; Lombardero *et al.*, 1992). Now we have analysed this possibility and studied the potential involvement of the carbohydrate in the allergenic recognition.

The olive pollen allergen mainly exists as two, glycosylated (20 kDa) and non-glycosylated (18.5 kDa), variants as occurs for other allergens such as *Api m I* from the bee *Apis mellifera* (Weber *et al.*, 1987). The glycosylated form of *Ole e I* has been identified as a concanavalin A-binding glycoprotein. Photometric assays and mass spectrometry analysis of the native allergen give a molecular mass of 1.0–1.3 kDa for the carbohydrate moiety. This value is lower than that deduced from the SDS-PAGE analysis: a mass of 1.5 kDa is the difference between the apparent molecular weights of the glycosylated and the non-glycosylated forms (Fig. 1b). This discrepancy may be explained by the contribution of the carbohydrate moiety to interactions of the glycosylated protein with the polyacrylamide matrix, resulting in an apparently increased molecular mass. The effect is frequently observed in the electrophoretic mobility of this kind of heteroproteins (Peterson, 1981).

The calculated molecular mass of the carbohydrate moiety accounts for six or seven monosaccharide units of the most common hexoses present in Asn-linked glycans found in vacuolar plant glycoproteins, such as *N*-acetylglucosamine, mannose, fucose or xilose (Laurière *et al.*, 1989). Moreover, the positive staining of the glycosylated allergen with biotinylated-Con A indicates the presence of mannose in the structure of the carbohydrate of *Ole e I* since that lectin is specific for this monosaccharide.

Only one carbohydrate group is present in the *Ole e I* molecule. In fact, only one sugar-containing tryptic peptide has been observed. The sequence analysis of this peptide indicates the presence of a Asn-glycosylated residue in a consensus sequence for *N*-glycosylation. The PNGase F treatment, specific for *N*-linked oligosaccharides, corroborates this kind of attaching site for the sugar since this enzyme completely removes the carbohydrate of *Ole e I*.

Enzymatic treatment of the allergen *Ole e I* with *N*-glycosidase F removes the sugar group and produces a polypeptide derivative that apparently conserves the original secondary structure. Moreover, both glycosylated and non-glycosylated variants of the allergen are recognized by the olive pollen hypersensitive human sera. Hence, the conformation of the polypeptide chain of *Ole e I* seems to be independent of the presence of the carbohydrate. Moreover, the deglycosylated allergen

maintains the specific IgE competition capability against the native protein, although with a diminished affinity. These results would indicate that the glycoprotein has allergenic determinants of both types, dependent and independent of the carbohydrate moiety.

On the other hand, the CD spectrum of the TFMS-treated allergen reveals a complete loss of the native conformation of the protein. This effect can be attributed to the chemical modification of many amino acid side chains, such as the oxidation of sulfur-containing residues, as described for the ovine luteinizing hormone (Edge *et al.*, 1981). This result would contrast with the observed data for fetuin (Sojar and Bahl, 1987) and *Parietaria judaica* allergen I (Polo *et al.*, 1991), which conserved the overall periodic secondary structures of the polypeptide chains after TFMS deglycosylation. However, fetuin does not contain Met nor Cys residues, which could be oxidized by this reagent. TFMS-treated *Ole e* I has lost the specific IgE-binding ability both in solution (ELISA assay) and under immobilized form (electrotransfer and immunostained), which is in agreement with the altered protein conformation, and suggests the existence of conformational allergenic determinants in the protein.

It is remarkable that the single tryptic glycopeptide of *Ole e* I is recognized by olive pollen sensitive human sera. This would indicate the existence of an epitope in this part of the molecule. Further analyses are required in order to ascribe the IgE binding ability to the amino acid structure and/or the carbohydrate moiety of the glycopeptide. However, the enzymatic deglycosylated allergen, which maintains its native conformation has partially lost the IgE recognition capability. Hence, the sugar group seems to influence the affinity for the IgE antibodies. Moreover, the molecular mass determined for this sugar is enough to induce antigenic responses (Feizi and Childs, 1985; Prenner *et al.*, 1992). In this regard, it is well known that oligosaccharides have a marked effect in the antigenicity of glycoproteins (Alexander and Elder, 1984) and many differentiation antigens of normal and neoplastic cells are oligosaccharide determinants rather than peptide determinants of glycoproteins (Feizi and Childs, 1985).

In summary, olive tree pollen allergen bears a small carbohydrate group that seems to be involved in the IgE recognition of the hypersensitive human sera. This group could be responsible for some of the immunological cross-reactions observed between olive pollen allergen and other proteins from oleaceae, even with allergens from unrelated pollens such as from ryegrass or couch grass (Baldo *et al.*, 1992), due to the relatively restricted diversity of the oligosaccharides from plant glycoproteins.

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measurements. We also thank Dr J. G. Gavilanes for helpful discussions and critical reading of the manuscript.

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EXHIBIT 3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of)
Jevnikar et al.)

Serial No. 08/617,874)

Filed: May 21, 1996)

For: METHODS AND PRODUCTS)
FOR CONTROLLING IMMUNE)
RESPONSES IN MAMMALS)

Examiner: M. Lubet

Group Art Unit: 1644

Date: June 1, 2000

SUPPLEMENTAL DECLARATION OF ANTHONY M. JEVNIKAR

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Anthony M. Jevnikar, do hereby declare and say as follows:

1. I am the co-inventor named in the above-identified application and I make this Declaration to supplement the information provided in the Declaration of Anthony Jevnikar dated September 27, 1999, and filed in this case on October 1, 1999 (hereafter "the first Jevnikar Declaration").
2. I have read and understood the Office Action mailed on December 2, 1999.
3. The Examiner has rejected claims 19 – 21, 28, 31 – 36 and 53 – 55 under 35 U.S.C. §103(a), as unpatentable over Lam et al. (U.S.P. 5,484,719), in view of Tobin et al., (U.S.P. 5,475,086) or Lernmark et al. (U.S.P. 5,792,620). The Examiner has also rejected claims 19 – 21, 28, 31 – 36, 42 and 53 – 55 as unpatentable over Lam et al. in view of Tobin et al. or Lernmark et al. in view of Carrington et al. (J. Virol, v. 64, p. 1590 (1990)). The Examiner has furthermore rejected claims 1, 5 – 10, 19 –

21, 28, 31 – 36 and 53 – 55 under 35 U.S.C. § 103 (a), as unpatentable over Lernmark et al., Tobin et al., Weiner et al., (U.S.P. 5,643,868) and Zhang et al. (PNAS, v. 88, p. 10253 (1991)) in view of Lam et al. The Examiner has also rejected claims 1, 5 to 10, 19 – 21, 28, 31 – 36, 41 – 42 and 53 – 55 under 35 U.S.C. 103 (a), as unpatentable over Lernmark et al., Tobin et al., Weiner et al., Zhang et al., and Lamont et al. (Immunology, v. 66, p. 595 (1989)), in view of Lam et al. and further in view of Carrington et al.

4. My appreciation of the teachings of Lam et al. and Tobin et al. were set out in paragraphs 4 to 10 of the first Jevnikar Declaration and my appreciation of Weiner et al. and Zhang et al. in paragraphs 11 – 12 of that Declaration.

5. Lernmark et al. discloses the cloning and expression of a DNA sequence encoding human islet cell GAD, and discusses the preparation and purification of recombinant GAD. Lernmark et al. also suggests that purified recombinant GAD can be used, either by parenteral injection or orally, to induce immunological tolerance to GAD autoantigen in patients predisposed to or already mounting an immune response to GAD.

Lernmark et al., however, clearly did not conceive of producing GAD recombinantly in transgenic plants and administering plant material containing expressed GAD orally to produce immune tolerance. Lernmark et al. teaches only mass culture production of recombinant GAD, followed by purification of the protein before administration. The entire discussion in this patent is within the context of in vitro recombinant expression, as evidenced, for example, by the following comments: "Host cells containing DNA constructs of the present invention are then cultured to produce the human islet cell GAD polypeptides. The cells are cultured according to standard methods in a culture medium... (column 8, lines 32 – 34); "selection of a medium appropriate for the particular cell line used is within the level of ordinary skill in the art" (column 8, lines 46 – 47); and "the human islet cell GAD produced according to the present invention may be purified by affinity

chromatography" (column 8, lines 59 – 60). Within this context there is provided, in the passage referred to by the Examiner, a "shopping list" of suitable host cell types, including plant cells, for in vitro expression.

It is stated at column 2, lines 64 to 67, that "For large scale production the expressed human islet cell GAD polypeptides can be isolated from the cells by, for example, immunoaffinity purification".

There is nothing in this reference to suggest the expression of an antigen such as GAD in a transgenic plant, followed by oral administration of plant material containing the expressed antigen to a subject.

6. Lamont et al. discloses the oral administration of ovalbumin to suppress a systemic immune response to that antigen.

There is nothing in this reference to suggest the expression of an antigen such as GAD in a transgenic plant, followed by oral administration of plant material containing the expressed antigen to a subject.

7. The Examiner notes in the Office Action of December 2, 1999, that she has considered the first Jevnikar Declaration and states that "Dr. Jevnikar alleges that GAD produced in plant materials had an unexpected property with respect to GAD produced recombinantly in E.coli, since a plant extract from a transgenic tobacco plant expressing GAD induces a greater in vitro proliferative response in T cells from animals immunized with GAD than does purified GAD obtained by purifying GAD recombinantly expressed in E.coli". The Examiner indicates that Exhibit 2 of the first Jevnikar Declaration lacks (1) data comparing purified GAD obtained by expression in E. coli with purified GAD obtained by expression in plants and (2) further lacks data comparing the T cell response to plant material (extract) containing expressed GAD with the T cell response to a control plant material (extract) from a transgenic plant not expressing GAD.

8. Firstly, with respect to point 1, the first Jevnikar Declaration asserts, at paragraph 14, that "the inventors have found unexpectedly that plant material containing plant-expressed mouse GAD protein stimulated a greater proliferative response of GAD-primed T cells than highly purified recombinant mouse GAD expressed in E. coli" (emphasis added). Furthermore, the claims are also directed to use of a plant material containing a plant-expressed antigen such as GAD. The first Declaration did not compare purified GAD obtained by plant expression with purified GAD obtained by E. coli expression; hence, no such comparative data are provided.
9. With respect to point 2, concerning data comparing T cell responses to plant material containing expressed GAD with T cell responses to control plant material from a transgenic plant not expressing GAD, the attached Exhibits 1 and 2 provide such data, as will be explained in more detail below.
10. Exhibit 1 is a summary of studies published by the inventors in Nature Medicine, v. 3, pp. 793 – 796 (1997), in a paper entitled "Transgenic plants expressing autoantigens fed to mice to induce oral immune tolerance", a copy of which was filed with the applicant's response dated January 5, 1998.
11. In accordance with the protocol set out in Exhibit 1, NOD mice were treated orally either with plant material (potato slices or tobacco leaves) containing expressed mouse GAD or with control plant material obtained from empty vector-transformed plants (potatoes or tobacco). After challenge with purified recombinant mouse GAD by foot pad injection, spleen cells were removed and examined for their in vitro proliferative response to purified recombinant mouse GAD (pur. rGAD) or a non-relevant antigen (ovalbumin). As seen in the RESULTS section of Exhibit 1, when animals were treated orally with control plant material from empty-vector transformed plants (PM), their spleen cells showed proliferation in response to purified mouse GAD but not to ovalbumin, indicating an immune response to GAD.

This immune response to GAD was suppressed in animals treated orally with plant material containing expressed mouse GAD (PM + GAD), whose spleen cells showed no proliferative response to purified mouse GAD. It was not possible to compare the effect of oral treatment with plant material containing expressed GAD with oral treatment with purified, E. coli-produced recombinant GAD in this experiment as we simply could not commit the resources required for the large scale fermentation and large scale purification required to make, in E. coli, and purify the levels of GAD (around 1mg/day/animal) required for oral administration to induce immune tolerance.

12. The studies described in Exhibit 1 show that plant material containing expressed GAD, administered orally, suppresses or reduces the immune response of a mammal to the autoantigen GAD; this effect is not seen after oral administration of control plant material from empty-vector transformed plants.

13. Exhibit 2 attached shows the results of an experiment similar to that described in Exhibit 2 of the first Jevnikar Declaration. For clarification, Figure 1 of attached Exhibit 2 (page 2/3) is in fact a linear scale Y axis version of the Figure previously presented with a logarithmic Y axis in the first Jevnikar Declaration. Figure 2 of attached Exhibit 2 (page 3/3) now shows control results obtained using a control plant material from plants which express an irrelevant control protein, MHC class II alpha chain protein (I-Ak). This control exposes the test animals to plant material in the absence of the antigen, GAD, to which they have been sensitized.

14. Both Figures 1 and 2 show that when mice are primed with plant material containing expressed GAD (PGAD), their T cells proliferate in response both to purified recombinant mouse GAD (MGAD) and to plant material containing expressed GAD (PGAD), but that the response to PGAD is much greater than the response to MGAD. Figure 2 shows that, in contrast, animals primed with plant material from a transgenic plant not expressing GAD (I-Ak) showed no response to

MGAD. These animals did, however, show a proliferative response to the plant material containing expressed GAD (PGAD); since these animals are not sensitized to mouse GAD, as indicated by the lack of response to MGAD, this response to PGAD indicates the T cell response to plant proteins or other constituents other than the expressed mouse GAD.

15. These results indicate that a small fraction of the T cell response to PGAD challenge seen in PGAD-primed animals is likely due to plant material components other than the expressed mouse GAD. The greater magnitude of T cell response of PGAD-primed animals to PGAD challenge, whether compared with the response of the same animals to MGAD (as in the Figures 1 and 2 attached) or with the response seen in MGAD-primed animals challenged with MGAD (as seen in Figure 1 attached), indicates that there is an unexpected synergistic enhancement of T cell activation when GAD-reactive T cells are primed with plant material containing expressed GAD.

Although the absolute levels of T cell proliferation observed vary somewhat from experiment to experiment, the highest levels of proliferation were always seen in cells from PGAD-primed animals challenged with PGAD.

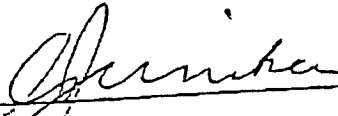
16. As indicated in the first Jevnikar Declaration, T cell activation is a prerequisite for the induction of immune tolerance, including oral tolerance. A T cell initially engages with an antigen when the antigen is presented to it by antigen presenting cells, leading to transmission of a first signal within the T cell. Depending on the presence or absence of further costimulatory signals, the T cell may progress to proliferation (immune response) or may become quiescent and unable to respond to the antigen (immune tolerance). Enhanced T cell activation, as seen after administration of plant material containing expressed GAD, can be expected to contribute to enhanced immune tolerance when subjects are treated orally with plant material containing expressed GAD, as described in this application.

17. Although the reason for the enhanced T cell activation seen after administration of plant material from transgenic plants containing expressed GAD is not presently known, it seems reasonable to postulate that other components of the plant material contribute to the observed synergistic effect. As noted at paragraph 16 of the first Jevnikar Declaration, lectins, which are common plant components, have been described as having immune-stimulating effects and may be contributing to the enhancement. It is interesting to note that plant lectins have been reported to induce secretion of IL-4 and IL-13 from human basophils and it is known that IL-4 is a key cytokine in the mediation of immune tolerance, through direction of the immune system towards a Th2 T cell response (Haas et al. (1999), Eur. J. Immunol., v. 29, pp. 918-927: copy enclosed).

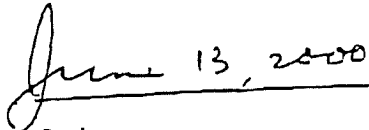
18. The teachings of Lam et al., and Tobin et al., whether or not supplemented by the teachings of Lernmark et al. and/or Carrington et al., cannot, in my opinion, be combined to arrive at the present invention, as discussed above; neither the teachings of Wiener et al. and Zhang et al. regarding the use of a native mammalian autoantigen, nor the additional teaching of Lamont regarding oral administration of ovalbumin to produce immune tolerance, can supplement the teachings of Lam, Tobin, Lernmark and Carrington so as to arrive at an appreciation that plant material containing plant-expressed mammalian antigens could be administered orally to produce immune tolerance or that such material would produce enhanced T cell activation.

19. I hereby declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false

statements may jeopardise the validity of the application or of any patent issued thereon.



Anthony M. Jevnikar



Date

EXHIBIT 1

Exhibit 1 to Supplemental Declaration of Anthony Jevnikar

Protocol

Step 1. NOD mice treated with oral antigen

- (i) plant material (potato slices or tobacco leaves) containing expressed mouse GAD (PM + GAD); or
- (ii) plant material control (empty vector-transformed plant) (PM)

Step 2. Antigen challenge by footpad injection

of purified recombinant mouse GAD produced in E. coli and purified (pur. rGAD)

Step 3. Removal of spleen cells from treated mice and in vitro stimulation of isolated spleen cells with antigen (pur. rGAD, ovalbumin or medium)

RESULTS

<u>Oral Treatment</u>	<u>Challenge Antigen</u>	<u>Stimulation Antigen</u> <u>(in vitro)</u>	<u>Results*</u> (spleen cell proliferation)
PM+GAD	pur.rGAD	pur.rGAD	no proliferation
PM+GAD	pur.rGAD	ovalbumin	no proliferation
PM+GAD	pur.rGAD	medium	no proliferation
PM(control)	pur.rGAD	pur.rGAD	Proliferation
PM(control)	pur.rGAD	ovalbumin	no proliferation
PM(control)	pur.rGAD	medium	no proliferation

* Summary of results shown in Figure 2 (a) of Nature Medicine paper.

EXHIBIT 2

Exhibit 2 to Supplemental Declaration of Anthony Jevnikar

Protocol

Mouse GAD67 was prepared recombinantly in E. coli and purified (MGAD). Mouse GAD67 was also expressed in transgenic tobacco, as described in this application, and a plant material containing expressed GAD67 (PGAD) was prepared by crushing leaves in buffer in a mortar and pestle and removing plant debris by centrifugation. Total protein concentration was determined by spectrophotometry.

A similar plant material was prepared from transgenic tobacco expressing MHC class II alpha chain protein (I-Ak).

500 µg of PGAD plant material containing 2.5 – 5 µg of GAD (50 µl volume) or 500 µg of I-Ak plant material (50 µl volume) was emulsified with an equal volume of incomplete Freund's adjuvant (IFA, Sigma Chemical Co., MO) and injected into each hind foot pad of 6 to 8 week old female non-diabetic NOD mice. IFA with PBS alone was used as control.

The mice were killed after 10 days, popliteal lymph nodes were removed and a single cell suspension was prepared. The cells were then cultured in 96-well flat bottom plates (Becton Dickinson, New Jersey) at 2×10^5 cells/well in RPMI (Biowhittaker, Maryland), supplemented with 10% FCS (GIBCO, Grand Island, NY), 10 mM HEPES, 5×10^{-5} M 2-ME and 1 U/ml Penicillin-Streptomycin. Cultures were stimulated with MGAD 20 µg/ml or PGAD 100 µg/ml, incubated for 72 hours, pulsed with 3H-thymidine (1 µCi/well; DuPont-NEN, Boston MA), harvested on glass fiber filter 16 h later and counted in a liquid scintillation counter (LKB Instruments, Gaithersburg, MD). The results are shown in the attached Figure. Cell proliferation is expressed as counts per minute +/- standard deviation.

Figure 1 of Exhibit 2, Supplemental Jevnikar Declaration

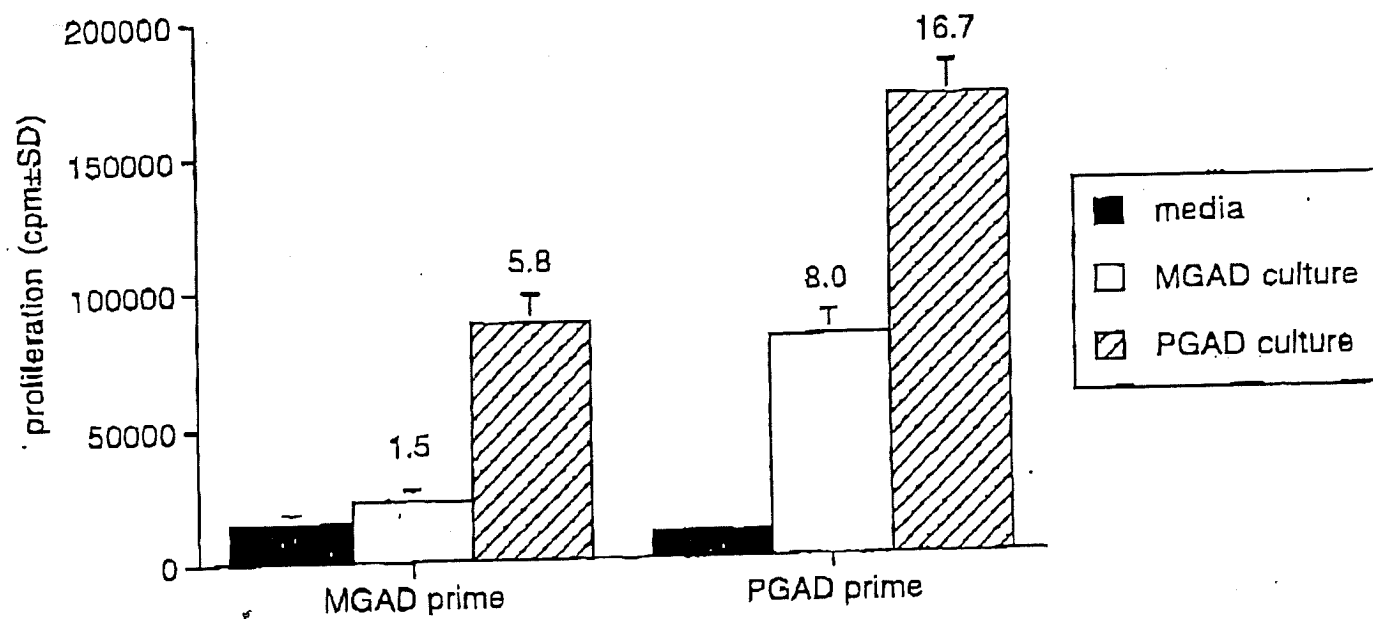


Figure 2 of Exhibit 2, Supplemental Jevnikar Declaration

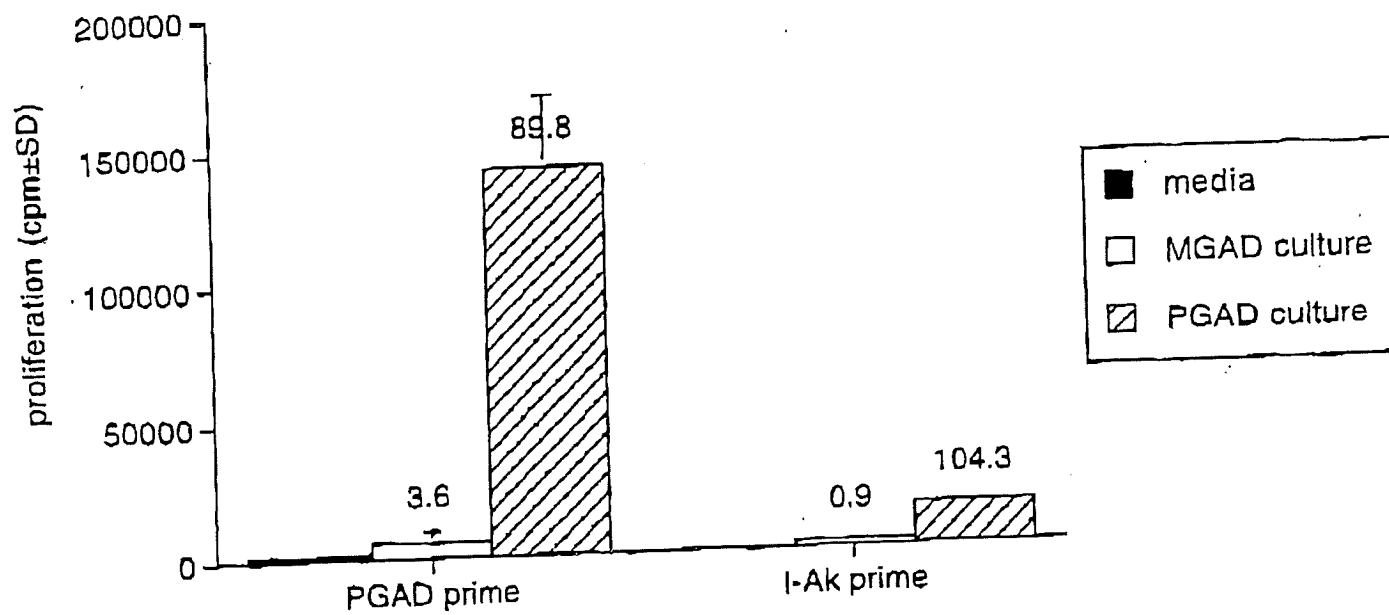


EXHIBIT 3

Dietary lectins can induce *in vitro* release of IL-4 and IL-13 from human basophils

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Dietary lectins, present in beans and other edible plant products, pose a potential threat to consumers due to their capacity to induce histamine release from basophils. In this study, we analyzed the capacity of 16 common, in particular dietary, lectins to induce human basophils to secrete IL-4 and IL-13, the key promoters of Th2 response and IgE synthesis. Several of the lectins, especially concanavalin A, lentil lectin, phytohemagglutinin, *Plisum sativum* agglutinin and *Sambucus nigra* agglutinin, triggered basophils to release IL-4 at concentrations of up to $1 \text{ ng}/10^6$ basophils. Lectins with high IL-4-inducing capacity also stimulated the release of IL-13 and histamine. Lectin-induced IL-4 and IL-13 release reached a maximum after 4–6 h and more than 18 h, respectively. Affinoblotting revealed that lectins with the capacity to induce mediator release bind to IgE, suggesting IgE binding as initial step of signal generation. In conclusion, several dietary lectins can trigger human basophils to release IL-4 and IL-13. Since lectins can enter the circulation after oral uptake, they might play a role in inducing the so-called early IL-4 required to switch the immune response towards a Th2 response and type I allergy.

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Key words: Basophil / IL-4 / Lectin

1 Introduction

The pathomechanism of IgE-mediated allergy is only partially understood. It is known that the early presence of IL-4 will direct an immune response towards the Th2 phenotype which is dominated by the production of IL-4, IL-5 and IL-13 [1] leading to elevated IgE synthesis. However, only preliminary knowledge exists about those cells providing early IL-4 as well as about the nature of the molecules inducing the production of early IL-4.

In principle, several cell types are able to produce IL-4 when appropriately stimulated: type 2 T cells, NK1.1 cells (in the mouse), eosinophils, mast cells and basophils [2, 3]. During a primary immune response the production of early IL-4 is most likely to be mediated by cells of the innate immunity, since at the time of primary contact with an immunogen mature antigen-specific cells

are not yet available. This excludes type 2 T cells from being the source of early IL-4. Among cells involved in innate immunity, basophils are favorite candidates, since they are rich sources of IL-4 [4–8], whereas eosinophils or mast cells produce moderate amounts of this cytokine [3, 7]. IL-4 is produced by basophils upon cross-linking of high-affinity Fcε receptors (FcεRI). Apart from IL-4, histamine, leukotrienes and IL-13 are released via this mechanism [4, 8–10].

Regarding the nature of putative molecules triggering the release of the early IL-4, proteases [11], superantigens [1] and lectins have to be considered. By cross-linking of cell surface glycoligands, various lectins can induce the secretion of cytokines by lymphocytes and monocytes. Lectins are substantial constituents of edible plant products such as beans and it is conceivable that ingestion of legume seeds can lead to IgE-mediated allergies. The major legumes involved in human food allergy are peanuts and soybeans, although allergic reactions have also been observed to peas, green beans, sweet lupins and lentils [12]. Moreover, the lectin Con A which is derived from the Jack bean (*Canavalia ensiformis*) was found to

[1 18899]

Abbreviations: FcεRI: High-affinity IgE receptor IMDM: Iscova's modified Dulbecco's medium

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induce [13] or to enhance [14] reaginic antibody formation in various mouse strains, underscoring the allergenic potential of plant lectins. Considering that Con A and several other lectins have been reported to promote histamine release from basophils and mast cells [15-17], presumably via cross-linking receptor-bound IgE [15, 18], we reasoned these lectins might also trigger the release of IL-4 and IL-13. To test this hypothesis, we studied the effect of 16 lectins mainly of plant origin on IL-4 and IL-13 production by human basophils *in vitro*.

2 Results

2.1 Dose- and time-dependent mediator release from basophils induced by Con A

Preliminary experiments had shown that IL-4 secretion by basophils can be induced by several plant lectins. To determine optimal conditions for stimulation, dose-response experiments were performed with Con A. Studying basophils from seven donors, maximal release of IL-4 was found at Con A concentrations around 1 μM (ranging from 0.2 to 5.0 μM) after an incubation time of 4 h. In the same set of experiments a comparable dose-response curve was obtained for the release of histamine, with an optimum around 0.2 μM (ranging from 0.04 to 1.0 μM) (Fig. 1). Pilot experiments with other lectins like *Phaseolus vulgaris* arthroagglutinin (PMA-E) and *Sambucus nigra* agglutinin (SNA) yielded similar results (not shown). For control purposes, basophils were stimulated in parallel with anti-IgE. The concentration of anti-IgE required for obtaining maximal IL-4 release ranged from 0.0024 to 0.012 μM . For maximal histamine release, it ranged from 0.0024 to 0.06 μM . Thus, in molar terms, maximal release of both mediators required higher concentrations of Con A than of anti-IgE. A possible explanation for this difference could be that lectins may interact with various cell surface molecules, whereas anti-IgE antibodies are directed to IgE exclusively. Although the bell-shaped dose-response curves differed between individuals, they revealed a common overlapping region at 1 μM . Since the reaction profile towards the lectins was rather broad, covering more than one order of magnitude of concentration, further comparative analysis was performed at the concentration of 1 μM regardless of differences in subunit composition of the lectins (Table 1), being aware that the maximal response may not be triggered in this comparative analysis.

Next, kinetics of Con A stimulation were assessed (Fig. 2). Similar to anti-IgE, Con A rapidly triggered release of IL-4 with the plateau level being reached after 4-6 h. In contrast, production of IL-13 continued after

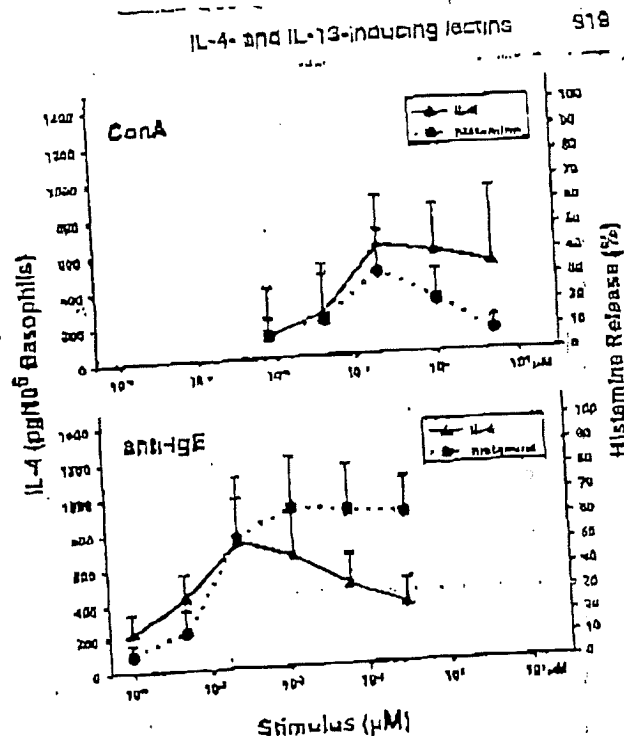


Figure 1. IL-4 and histamine are released in a dose-dependent manner upon stimulation of human basophils with Con A and anti-IgE. Basophil preparations from human donors ($n = 7$) were incubated with various concentrations of Con A and anti-IgE. Supernatants were recovered after 4 h for mediator determination (mean \pm SD).

this time. This kinetic pattern is in agreement with other reports [8, 9, 18]. Although not maximal, IL-13 levels were clearly elevated after 4 h. We therefore chose a standard incubation time of 4 h for assessing the mediator-releasing capacity of the lectins studied.

2.2 IL-4 production upon stimulation with Con A correlates with basophil purity

Since IL-4 is produced not only by basophils, cells contaminating the basophil preparations could be an additional source of lectin-induced IL-4. To address this issue, three basophil preparations (purity between 82 and 84 %) were remixed at various proportions (1:2 dilutions) with autologous PBMC and stimulated in parallel with Con A, anti-IgE or ionomycin (Fig. 3). Upon all three stimuli, IL-4 production correlated with the degree of basophil purity. These results strongly suggest that Con A-induced IL-4 is essentially derived from basophils and not from other cell types.

Table 1. Molecular masses, subunit composition and carbohydrate specificities of the lectins tested in this study

Lectin	Abbreviation	kDa	Subunits	Carbohydrate specificity
<i>Arachis hypogaea</i> (peanut) agglutinin	PNA	120	4	Gal β 1-3 GalNAc α / β - > terminal β -Gal
<i>Anticarpus integrifolia</i> agglutinin (Jacalin)	AIA	85	4	Gal β 1-3 GalNAc α - and α -Gal
<i>Canavalia ensiformis</i> agglutinin	Con A	102	4	Terminal α -D-Man/ α -D-Glc, branched mannose, core of complex biantennary N-glycans
<i>Codium fragile</i> agglutinin	CFA	60	4	GalNAc
<i>Datura stramonium</i> agglutinin	DSA	86	2	Terminal or internal (GlcNAc β 1-4 GlcNAc) $_n$ = Gal β 1-4 GlcNAc residues
<i>Erythrina corallodendron</i> agglutinin	ECA	60	2	Gal β 1-4 GlcNAc, Gal β 1-4 Glc, and α -Gal
<i>Glycine max</i> (soybean) agglutinin	SBA	110	4	α , β -GalNAc > α , β -Gal
<i>Helix pomatia</i> agglutinin	HPA	79	6	α -GalNAc
<i>Lens culinaris</i> agglutinin	LCA	49	2	α -D-Man and α -D-Glc residues, fucosylated core region of bi- and triantennary N-glycans
<i>Phaseolus vulgaris</i> erythroagglutinin	PHA-E	128	4	Bisected N-glycans, Man α 1-6-linked part of tri- or tetraantennary N-glycans
<i>Phaseolus vulgaris</i> leucoagglutinin	PHA-L	126	4	Gal β 1,4 GlcNAc β 1,2 [Gal β 1,4 GlcNAc β 1,6] Man
<i>Pisum sativum</i> agglutinin	PSA	49	4	α -D-Man, α -D-Glc, fucosylated core region of bi- and triantennary N-glycans
<i>Ptilota plumosa</i> agglutinin	PPA	55	-	Terminal α -D-Gal residues
<i>Ricinus communis</i> agglutinin (120 kDa)	RCA ₁₂₀	120	4	Terminal β -D-Gal residues, α 2-6 sialylation tolerated
<i>Sambucus nigra</i> agglutinin	SNA	140	4	NeuNAc α 2-8 Gal/GalNAc
<i>Triticum vulgare</i> (wheat germ) agglutinin	WGA	38	2	GlcNAc β 1-4 GlcNAc β 1-4 GlcNAc > GlcNAc β 1-4 GlcNAc > GlcNAc > sialic acid

2.3 IL-3 enhances Con A-induced IL-4 production by basophils

IL-4 production by basophils following cross-linking of Fc ϵ R1 is enhanced in the presence of IL-3 [4]. To assess the effect of this cytokine on lectin-induced IL-4 production, basophils from eight donors were stimulated with Con A in the presence and absence of IL-3. As shown in Fig. 4, Con A- and anti-IgE-induced IL-4 production were similarly enhanced by IL-3, suggesting an involvement of Fc ϵ R1 in Con A-induced IL-4 production. Since IL-3 per se stimulates the production of IL-13 by basophils at levels as high as those induced by Fc ϵ R1 cross-linking [8, 10, 19], the following comparative analysis was performed without addition of IL-3.

2.4 Lectins differentially induce the release of IL-4 and IL-13 from basophils

A panel of 16 lectins with a broad range of sugar specificities (see Table 1 for compilation and abbreviations) was investigated for their capacity to induce the release of IL-4 and IL-13 from basophils. The effect of each lectin was studied on basophils from at least nine different donors. Fig. 5 shows results of a determination of IL-4, IL-13 and histamine release for each donor in parallel. Remarkably, the lectins differed considerably in their capacity to induce the release of IL-4 from basophils. Con A, *Lens culinaris* agglutinin (LCA), PHA-E, *Pisum sativum* agglutinin (PSA) and SNA effectively induced IL-4, whereas other lectins like *Anticarpus integrifolium* agglutinin (AIA), *Codium fragile* agglutinin (CFA), soy bean agglutinin (SBA), *Helix pomatia* agglutinin (HPA) and *Ptilota plumosa* agglutinin (PPA) had little or no effect. The former lectins induced basophils of nearly all donors to produce IL-4 at concentrations equivalent to

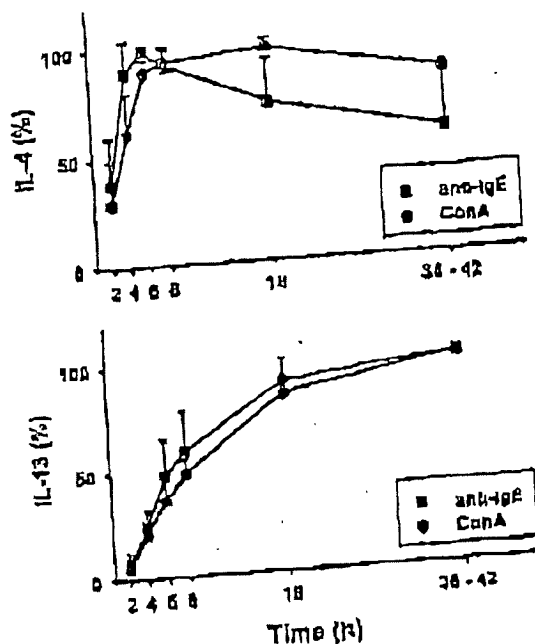


Figure 2. Kinetics of IL-4 and IL-13 release from human basophils after stimulation with Con A and anti-IgE. Basophils (purity between 64 and 70 %) from three donors were stimulated with 1 μ M Con A and 0.4 μ g/ml rabbit anti-IgE for various time intervals (values in percent of maximum cytokine release; mean \pm SD).

those induced by anti-IgE. The cytokine induction by *Ricinus communis* agglutinin (RCA₁₂₀), a lectin which has been reported to bind IgE and to trigger histamine release from basophils of the majority of donors [16], was not evaluable due to its nonspecific binding to anti-cytokine antibodies in the cytokine ELISA. When we compared lectin-induced IL-4 production of basophils from type-I allergic individuals without actual symptoms ($n = 8$) and nonallergic donors ($n = 14$), we did not observe significant differences (Mann-Whitney test; data not shown), except for SBA ($p < 0.005$), but this lectin had a low absolute effect. In addition to IL-4, secretion of IL-13 and histamine was also induced by several lectins. The profiles of mediator release were similar, suggesting a common release mechanism for all three mediators.

2.5 Putative interaction between various lectins and IgE (PS)

Since IgE is highly glycosylated with a carbohydrate content of 11.7 %, as shown for myeloma IgE (ND), we assessed the possible role of IgE in lectin-induced mediator release. The structure of the glycan components of

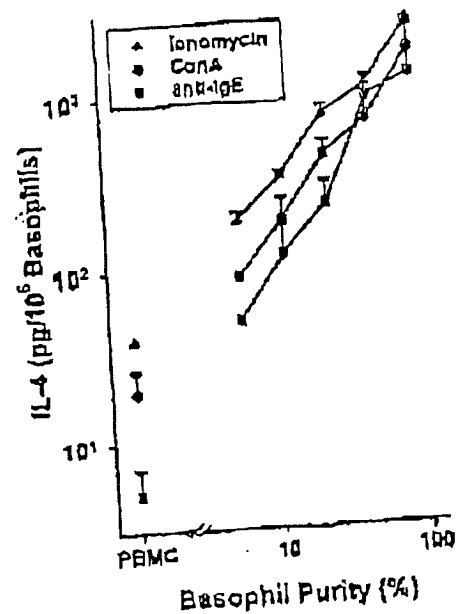


Figure 3. IL-4 production depends on the purity of the basophil preparation. Basophils (purity 84 %) were mixed back at 1:2 step dilutions with autologous PBMC and stimulated with 1 μ M Con A, 0.4 μ g/ml rabbit anti-IgE, or 1 μ M ionomycin (mean \pm SD of duplicate determinations; shown is one representative out of three experiments).

myeloma IgE (PS) has been determined [20, 21]. Two N-linked glycan types have been described on this IgE, the complex biantennary and the high-mannose type. Considering the carbohydrate specificities of the lectins under study (Table 1), this structural information allows to depict putative lectin-binding sites on IgE. High-

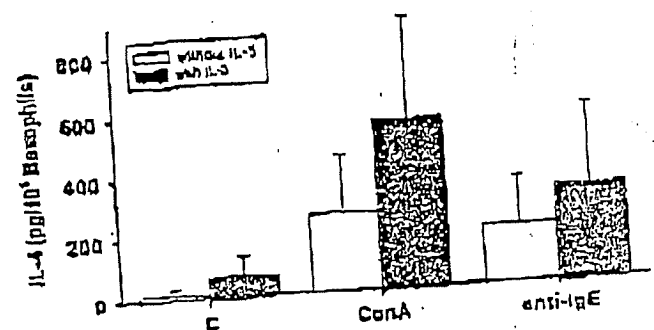


Figure 4. IL-3 enhances Con A-induced IL-4 production by basophils. Basophils from human donors ($n = 9$) were incubated with 1 μ M Con A, 0.4 μ g/ml rabbit anti-IgE, or medium (C) in the absence or presence of 10 ng/ml IL-3. Supernatants were recovered after 4 h for determination of IL-4 (mean \pm SD).

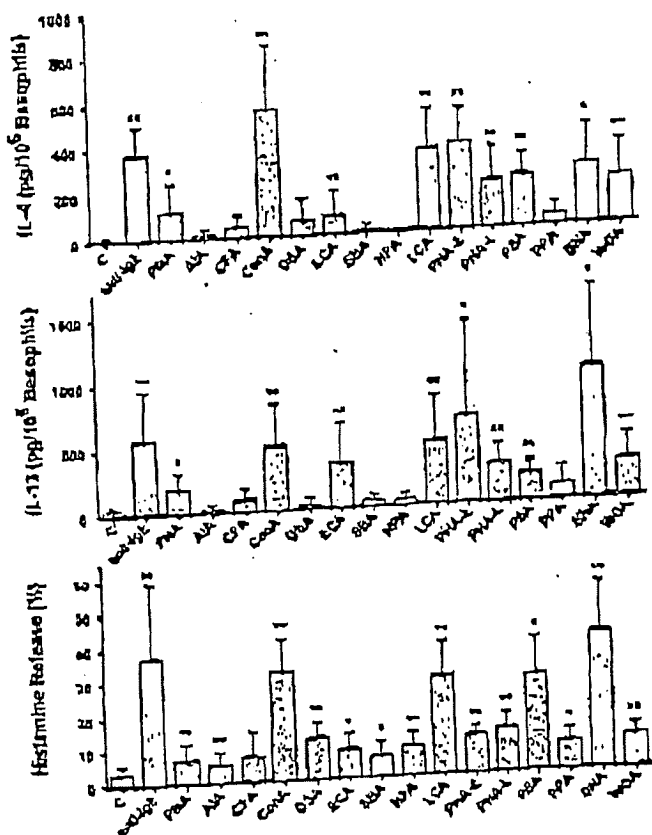


Figure 5. Differential capacity of plant and invertebrate lectins to trigger basophils to release IL-4, IL-13 and histamine. Basophils from human donors ($n = 9-11$) were stimulated in parallel with the lectins under study ($1 \mu\text{M}$). The supernatants were recovered after 4 h for mediator determination (mean \pm SD). Results significantly differing from the medium control (C) are indicated by asterisks: * $p < 0.02$, ** $p < 0.008$ (Wilcoxon matched-pairs signed-rank test).

affinity sites for six of the 16 lectins [Con A, *Datura stramonium* agglutinin (DSA), *Erythrina corallodendron* agglutinin (ECA), RCA₁₂₀, SNA and wheat germ agglutinin (WGA)] are present on the complex biantennary glycan (Fig. 6) and for two further lectins (LCA and PSA, in addition to Con A) on the high-mannose glycan (not shown). The reactivity of PHA-E and *Phaseolus vulgaris* leucoagglutinin (PHA-L), which have extended binding sites, is not unambiguously predictable. The residual six lectins [AIA, CFA, SBA, HPA, PPA and peanut agglutinin (PNA)] do not find putative binding sites (PNA only, if the glycan is desialylated; see Fig. 6). There are currently no data on the precise glycan structure of polyclonal IgE. However, it is noteworthy that the basic structure of the N-linked glycans is quite uniform within [22] and between [23] Ig isotypes and that – across species – it essentially corresponds to that found in IgE (PS). Therefore, the carachy-

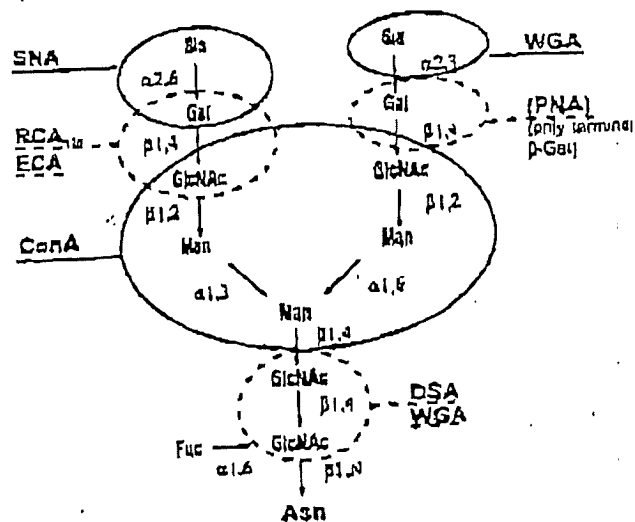


Figure 6. Lectin-reactive sequence elements on the complex biantennary N-glycan of IgE (PS). For individual lectins the corresponding binding partners are indicated. Sia, Gal, GlcNAc, Man, Fuc and Asn represent sialic acid, galactose, N-acetyl-glucosamine, fucose and asparagine, respectively.

arate composition of IgE (PS) may essentially reflect that of polyclonal IgE.

2.6 Binding of lectins to myeloma IgE as assessed by affinity blotting

To assess whether the mediator-releasing capacity of the lectins matches their ability to bind IgE, affinity blot experiments were performed. Four highly purified myeloma IgE preparations [IgE (PS), IgE (T+H), IgE (PE), and IgE (VL)] were separated by SDS-PAGE, blotted onto nitrocellulose membrane, and incubated with the labeled lectins. Apart from IgE, no significant lectin-binding bands were detected (data not shown). In Fig. 7 the blots of the four IgE preparations are aligned for comparison. The overall binding pattern of lectins is similar between the myeloma IgE. Strong binding was found for Con A, PHA-E, RCA₁₂₀ and SNA, moderate binding for DSA, ECA, LCA, PHA-L, PSA and WGA, and no or very weak binding according to the theoretical considerations was observed for PNA, AIA, CFA, SBA, HPA and PPA. It should be mentioned that, depending on the environment, the same glycan sequence can have different conformations resulting in different affinity to a lectin [24]. It is thus possible to detect only minor lectin reactivity despite presentation of a potentially suitable lectin-binding site. Noteworthy, from the ten IgE-reactive lectins, eight triggered cytokine release from basophils: Con A, ECA, LCA, PHA-E, PHA-L, PSA, SNA and WGA (Fig. 5). The other two were DSA

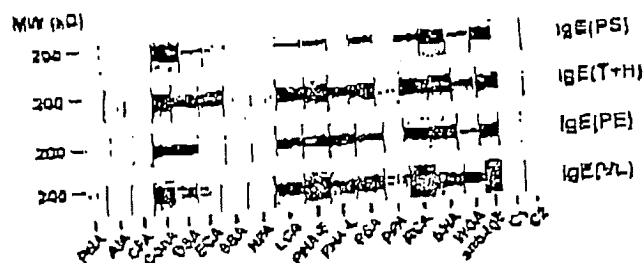


Figure 7. Binding of labeled lectins to IgE myeloma proteins as determined by affinoblotting. Highly purified IgE [IgE (PS), IgE (T+H), IgE (PE), and IgE (VL)] was separated by SDS-PAGE, blotted onto nitrocellulose, and incubated with the labeled lectins under study. Controls: anti-IgE; alkaline phosphatase-labeled monoclonal anti-IgE, C1; alkaline phosphatase-labeled anti-digoxigenin Ab, C2; alkaline phosphatase-labeled streptavidin.

and RCA₁₂₀. Despite binding, PSA had only minimal cytokine-releasing capacity. This might be due to a different accessibility of the glycan chains of native cell-bound IgE versus denatured blotted IgE. RCA₁₂₀ was not included in the results due to high background binding in the cytokine ELISA. From the six lectins that did not bind to IgE upon affinoblotting, five (AIA, CFA, SBA, HPA and PPA) had little or no mediator-releasing effect. One lectin, PNA, although negative by affinoblotting, triggered mediator release in at least three out of nine donors. PNA only interacts with terminal galactose residues, therefore it is not expected to bind to the sialylated IgE (PS). Since none of the respective donors had a peanut allergy or serum IgE reactive with peanut extract (data not shown), this suggests the presence of desialylated IgE in respective donors. Taken together, the observed association between functional activity and IgE reactivity of the lectins suggests a role of IgE in the mechanism of lectin-induced mediator release.

3 Discussion

Here we report that certain plant lectins, in contrast to others, can trigger human basophils to release IL-4 and IL-13. Of the 18 lectins studied, Con A, LCA, PHA-E and SNA induced IL-4 levels as high as those obtained by stimulation with anti-IgE antibodies. Lectins with high IL-4-inducing capacity triggered the release of IL-13 and histamine, whereas lectins with low or missing IL-4-inducing capacity did not or minimally induce the other mediators. Our data correlate well with the work of Shibasaki et al. [16], who investigated the effect of 12 lectins on histamine release from human basophils. Of the eight lectins that were studied by both groups, only one lectin,

PHA-L, is nearly negative according to Shibasaki et al., whereas in our hands it induced the release of all three mediators.

The mechanism by which lectins trigger basophile to release mediators is not completely understood. Considering the great diversity of sugar specificities recognized by different lectins and the multiplicity of glycosylated surface molecules on basophils, several, not mutually exclusive mechanisms may be operative. Since IgE is a glycoprotein, lectins might cross-link IgE bound to FcεRI [15, 16]. Remarkably, FcεRI is also a glycoprotein [25]. Thus, lectins might directly cross-link this receptor. Lectins could also engage other surface molecules on basophils, like the CSa receptor (CD88), which potentially mediates IL-4 and IL-13 induction upon ligation in the presence of IL-3 [10]. Since IL-4 production upon cross-linking of FcεRI on basophils is enhanced by IL-3 [4], we examined whether IL-3 had an analogous effect on lectin-induced IL-4 production. Our findings (Fig. 4) are compatible with a direct or indirect involvement of FcεRI in Con A-induced IL-4 production.

Interestingly, when comparing the mediator-inducing capacity of the lectins (Fig. 5) with their putative binding to IgE (PS), seven of eight evident binding candidates (see Sect. 2.5 and Fig. 6) had pronounced mediator-inducing potential, whereas one (PSA) was very weak. For this lectin accessibility to the stem region of N-glycans may be rather low *in situ*. In contrast, there was minimal or no release of mediators in response to lectins, which had no putative specificity to oligosaccharide components of IgE. To assess the actual binding of the lectins to IgE, affinoblotting experiments were performed. Their results were in agreement with the theoretical considerations: putatively reactive lectins actually bound to the blotted myeloma IgE. Taken together, these results demonstrate that both theoretical and actual binding of lectins to IgE parallels their capacity to induce release of IL-4, IL-13 and histamine, suggesting IgE to be involved in lectin-induced mediator release from basophils. To further clarify this mechanism, basophils depleted of surface IgE and basophils resensitized with IgE were stimulated with lectins. However, the results were inconsistent (data not shown). Similarly, when basophils were preactivated via anti-IgE in the absence of Ca²⁺ ions to desensitize the FcεRI-mediated activation pathway, the results were not evaluable, since this treatment did not only down-regulate IgE-mediated, but also ionomycin-induced IL-4 release (data not shown). We expect a further elucidation of the mechanism of lectin-induced IL-4 release from the use of basophil or mast cell lines.

Do lectins trigger basophils to release IL-4 and IL-13 also *in vivo*? There is circumstantial evidence for such an

effect. Lectins are present in many plant-derived foods, especially in legume seeds [26]. Thus, we may ingest lectins at considerable quantities depending on the individual diet preferences. Although most food lectins are apparently harmless, some cause clinical illness. Well studied is the intoxication by raw or improperly cooked red kidney beans (*Phaseolus vulgaris*) [27] which seems to be mainly due to PHA, the major lectin of this legume species [28]. In rodents the amount of systemically absorbed PHA may reach 5–10% of that given intragastrically [28]. While most of the lectin is bound to serum glycoproteins, a small proportion binds to blood cells [28], which suggests that dietary lectins may come into close contact with basophils also *in vivo*. In addition, apparently nontoxic food lectins like tomato lectin or PNA have been shown to be absorbed into the mucosa following ingestion [29, 30]. Finally, in certain mouse strains Con A was found to be allergenic [13] or to enhance reaginic antibody formation [14].

In conclusion, several dietary lectins are capable to rapidly trigger IL-4 and more slowly IL-13 release from human basophils *in vitro*. Considering that we may ingest lectins at substantial quantities with our food, this argues in favor of the possibility that lectins might play a role in the initiation of an allergic inflammation by provoking IL-4 and IL-13 in the early phase of the immune response.

4 Materials and methods

4.1 Reagents and materials

All label-free lectins (see Table 1) were from Sigma, Deisenhofen, Germany. Labeled lectins (digoxigenin-labeled PNA, Con A, DSA, PHA-L, ACA_{12a}, SNA, and WGA) as well as phosphatase-labeled anti-digoxigenin antibody were from Boehringer Mannheim, Mannheim, Germany; biotinylated AIA, HPA, LCA and PSA were from Sigma. HBSS, leucocyte modification medium (IMDM), penicillin and streptomycin were from Gibco-BRL, Paisley, GB; FCS from Boehringer Mannheim; Ficoll from Biochrom, Berlin, Germany; Agarose-IEF and Percoll from Pharmacia, Freiburg, Germany; M450-anti-CD2, -anti-CD14, -anti-CD19 immunomagnetic beads from Dynal, Hamburg, Germany; recombinant human (h) IL-4 (No. 2131-01) from Genzyme, Cambridge, MA; hIL-13 (No. 213-IL005) from R&D Systems, Abingdon, GB; polyclonal rabbit anti-human IgE antibody (No. AD94) from Dako, Glostrup, Denmark; mouse anti-human IL-4 (clone BD4-8), biotinylated rat anti-human IL-4 (clone MP4-25D2), rat anti-human IL-13 (clone JES10-SA2), and biotinylated rabbit anti-human IL-13 (No. 2D6 92 D) were from Pharmingen, San Diego, CA; streptavidin-alkaline phosphatase from Jackson Immuno Research, West Grove, PA; the mixture of two myeloma IgE, designated in this study as IgE (T+H) (No. 10224) from Scripps Laboratories, La

Jolla, CA; servalgates 3–10 and servalgates 4–6 from Serva, Heidelberg, Germany; nitrocellulose membrane (0.45 µm) from Schleicher & Schuell, Dassel, Germany; biotin-N-hydroxysuccinimide ester, bovine insulin (I-1822), BSA (A-7906), human transferrin (T-2252), ionomycin, and p-nitrophenylphosphate from Sigma; sterile round-bottom 96-well tissue culture plates from Nuncion and Maxisorp F96 microtiter plates from Nunc, Roskilde, Denmark. Alkaline phosphatase-labeled monoclonal anti-IgE was kindly provided by Allergopharma (Reinbek, Germany) and mIL-3 was kindly provided by Kinn (Gunma, Japan). Purified myeloma IgE were gifts from Dr. K. Ishizaka (La Jolla Institute for Allergy and Immunology, La Jolla, CA); IgE (PS), Dr. V. Krausz (Charles University, Pilsen, Czech Republic); IgE (VL), and Dr. C. G. M. Magnusson (Karolinska Institute, Stockholm, Sweden); IgE (PE).

4.2 Blood donors

Blood donors were healthy nonallergic and type-I allergic volunteers without actual symptoms. The diagnosis of allergy was based on clinical history (allergic rhinitis, allergic asthma), on a positive skin prick test response to specific allergens (with the majority of individuals reacting to grass pollen) and on specific IgE antibodies ≥ 17.5 kU/l as measured by the CAP system (Pharmacia, Freiburg, Germany).

4.3 Purification of basophils

Basophils were purified as described previously [6]. Briefly, 220 ml freshly drawn EDTA-blood was centrifuged on a Ficoll/Percoll discontinuous gradient. Interphase cells were separated by counter-current centrifugal elutriation, fractions with the highest basophil purity were pooled and subjected to negative selection with immunomagnetic beads. The purity of the final basophil preparations used in this study was $59.2 \pm 13.5\%$ (mean \pm SD, range 20–84%) with contaminating cells consisting mainly of monocytes and occasionally few lymphocytes. The mean yield was $4.2 \pm 2.5 \times 10^6$ basophils (mean \pm SD; range $1.1 - 18.5 \times 10^6$ basophils).

4.4 Lectin stimulation of basophils

Basophil preparations were resuspended at a concentration of 1×10^6 basophils/ml in IMDM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml bovine insulin, 50 µg/ml human transferrin and 10% FCS. They were exposed to the different reagents at the following concentrations: lectins (if not stated otherwise): 1 µM, mIL-3: 10 ng/ml; rabbit anti-hIgE: 0.4 µg/ml; ionomycin: 1 µM; as a control, cells were exposed to medium only. Cells were kept in sterile round-bottom 96-well microtiter plates at 37 °C in a humidified atmosphere with 5% CO₂. Supernatants were harvested after 4 h and stored at -20 °C.

4.5 Determination of IL-4, IL-13 and histamine

Cytokines were determined using a sandwich ELISA. The buffer was 0.1 M Tris/HCl, 0.1 M NaCl, 2.5 mM MgCl₂, pH 7.5 (referred to as Tris). Microtiter plates (Maxisorp F96) were coated overnight at 4 °C with mouse anti-IL-4 mAb or mouse anti-IL-13 mAb (1:4000 or 1:1000, respectively, in Tris). Following incubation for 90 min with samples or serial dilutions of rIL-4 or rIL-13, biotinylated rat anti-IL-4 mAb or biotinylated rat anti-IL-13 mAb (1:4000 or 1:1000, respectively, in Tris/0.05 % Tween-20 (Tris-T) containing 0.5 % BSA) was added for 60 min, after which the plates were incubated with streptavidin-alkaline phosphatase (1:1000 in Tris-T/0.5 % BSA) for 60 min. Then the chromogen solution (1 mg/ml p-nitrophenylphosphate in 0.1 M Tris/HCl, 0.1 M NaCl, 5 mM MgCl₂, pH 9.5) was added. The absorption was read at 405 nm. Between all incubation steps, plates were washed 7 x with Tris-T. Both assays had a sensitivity of 50 pg/ml and an upper limit of 3.3 ng/ml.

Histamine content of both supernatants and cell pellets (lysed in 4 % perchloric acid) was assayed using a spectrofluorimetric autoanalyzer (Bran & Luebbe, Norderstedt, Germany). The percentage histamine release for each tube was determined from the total histamine content in the sum of pellet and supernatant tubes. Histamine release without corrections is shown.

4.6 Biotinylation of lectins

Most lectins were commercially available in labeled form. Exceptions were CFA, ECA, SBA, PHA-E and PPA which were biotinylated according to the method previously described [31]. The lectin-containing solutions (5 mg/ml) were dialyzed overnight at 4 °C against PBS and adjusted to pH 8.5 with 5 % (w/v) sodium carbonate. Biotin-N-hydroxysuccinimide ester dissolved at 1 mg/ml in dimethyl-sulfoxide was added at a ratio of 0.1 mg of biotin-N-hydroxysuccinimide ester per 0.6 mg of lectin. The mixture was incubated for 4 h at room temperature and dialyzed against PBS pH 7.2 overnight at 4 °C. The biotinylated lectins were divided into aliquots and stored at -20 °C. Affinity blot titration on serum glycoproteins revealed that these solutions (final lectin concentration approximately 2 mg/ml) could be diluted at least 1:4000 (CFA, PPA) or 1:40000 (ECA, SBA, PHA-E) to give a clear and sugar-inhibitable signal. This indicates that the labeled lectins were functionally active.

4.7 SDS-PAGE and affinity blotting

The constituents of four myeloma IgE preparations (PS, T+H, PE and VL) were separated by SDS-PAGE according to the method of Laemmli [32] at a concentration of 5 µg/cm using 7.5 - 17 % gradient gels. The proteins were subsequently transferred onto nitrocellulose membrane by semi-

dry blotting for 30 min at 0.8 mA/cm² [33]. After blotting, the nitrocellulose membrane was incubated in 0.1 M Tris-buffered saline (TBS, pH 7.4) containing 0.05 % (v/v) Tween-20 for blocking free protein binding sites. For detection of carbohydrate molecules, membranes were incubated overnight at room temperature with solutions of the labeled lectins: PNA (1:100), Con A (1:10000), DSA (1:1000), PHA-L (1:1000), RCA₁₁₉ (1:10000), SNA (1:1000) and WGA (1:500; all digoxigenin-labeled); AIA (1:10000), CFA (1:1000), ECA (1:20000), HPA (1:50), LCA (1:2500), PSA (1:500), PPA (1:1000), SBA (1:6000) and PHA-E (1:2000; all biotinylated). Binding of digoxigenin-labeled lectins was visualized by alkaline phosphatase-labeled streptavidin (1:10000) and binding of biotinylated lectins by alkaline phosphatase-labeled streptavidin (1:30000). These secondary reagents did not bind directly to IgE, as shown by respective negative controls. Sugar specificity of lectin binding to IgE was ascertained by competitive inhibition experiments in the presence of asialofetuin (0.65 mg/ml), mannose (0.2 M), lactose (0.2 M), and glycophorin (0.25 mg/ml). Alkaline phosphatase-labeled monoclonal anti-IgE (1:2000) was used for detection of IgE.

Acknowledgements: We thank J. Kleine-Tebbe, E. T. Rietchel, A. Butz and M. Ernst for helpful discussion as well as G. Brinkmann, A. Gronow and B. Brockmann for their excellent technical support.

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EXHIBIT 4

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)	
Anthony Jevnikar et al.)	Group Art Unit: 1644
Application No.: 10/005,073)	Examiner: GERALD R EWOLDT
Filed: December 7, 2001)	Confirmation No.: 8806
For: METHODS AND PRODUCTS FOR)	
CONTROLLING THE IMMUNE)	
RESPONSES IN MAMMALS)	

DECLARATION OF ANTHONY M. JEVNIKAR

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Anthony M. Jevnikar, do hereby declare and say as follows:

1. I am a co-inventor named in the above identified application.
2. I have read and understood the Office Action mailed on October 8, 2004 (the "Office Action").
3. In the Office Action, the Examiner has rejected claims 52, 59-61, 63, 69-91 and 95 "under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention." OFFICE ACTION at 2.

The Examiner has argued that the claimed invention is not adequately enabled because "... results in mouse models do not correlate with results in humans. Whereas tolerance has been repeatedly induced in mice, the identical/equivalent methods have not worked in humans". OFFICE ACTION at 2.

This rejection was discussed during an Examiner interview on January 18, 2005 in which I was present. During the interview, the Examiner had indicated that three documents referred to in the last Office Action dated February 10, 2004, namely, Marketletter 1999; Goodnow 2001; and WO 02/53092 supported the notion that it has been repeatedly shown that results in mouse models do not correlate with results in humans. Furthermore, the Examiner asserted that there is in fact a lack of efficacy and indeed, danger, of administering antigens to humans in attempts to induce tolerance.

Firstly, the Marketletter 1999 Newsletter appears to be a newspaper publication and may contain errors as it is not a scientific publication and is thus not peer reviewed. For this reason alone, I would not consider this document to be a reliable scientific document. In any event, this newsletter clearly states that Colloral had failed in Phase III development not because it had no effect or because it posed any danger to human health, but rather because the effect it did have in human clinical trials was not statistically significant enough to warrant further spending on late phase clinical testing.

The document by Goodnow et al., is an overview of self-tolerance pathways and is not a description of a specific experiment that is designed to induce tolerance. In fact, the last sentence of the abstract that the Examiner cited is directed to the strategies noted earlier in the abstract which are not the same strategies as currently claimed and

described in the subject patent application for which I am a co-inventor. The statement in Goodnow et al. regarding the unpredictability, is in fact directed against the use and mechanism of action of corticosteroids.

WO 02/053092 discloses a method of inducing immune tolerance to plaque associated molecules such as LDL, beta-2-GPI and HSP in humans. The Examiner refers to page 23 of the description to assert that the inventors of the '092 PCT publication conclude that "oral and mucosal tolerance cannot be deduced from antigenic activity in conventional immunization, or even *in vitro* results and must result from extensive empirical experimentation". It should be noted that this conclusion in the '092 PCT publication is with respect to a summarized description of prior art that does not involve any oral antigen presentation by the oral administration of plant material that contains the antigen as is the case in the presently claimed invention.

Despite the prior art summary discussion on page 23 of the '092 PCT publication, the inventors of the '092 PCT publication proceeded to overcome the described short coming of the prior art with their invention. The '092 PCT publication ultimately shows that immune tolerance can be accomplished.

It is clear that these documents do not address the method of the subject invention for which I am a co-inventor which is a method for suppressing or reducing an immune response by administration of an antigen as is expressed and contained in a plant material.

I further disagree with the Examiner's statement that oral tolerance has never before been successfully demonstrated in humans. Oral tolerance to protein antigens

has in fact been demonstrated in humans after antigen ingestion. Attached hereto as Exhibit A is a copy of a paper by Husby et al., Oral Tolerance in Humans, The American Association of Immunologists, 1994, pp. 4663-4670, that clearly demonstrates oral tolerance in humans. More specifically, this work demonstrates the suppression of peripheral T cell responses to the protein antigens KLH, after oral administration of KLH.

Oral tolerance has also been demonstrated by the administration of Type I collagen to autoimmune patients with Systemic Sclerosis. This was demonstrated to induce significant reductions in levels of INF γ and IL-10 in stimulated peripheral blood lymphocyte culture supernatants, indicating that T cell immunity to collagen was decreased by oral collagen administration. This study is described in McKown et al., Arthritis & Rheumatism, Vol. 43, No.5, May 2000, pp. 1054-1061 (Exhibit B).

These two representative references support the fact that oral tolerance can be induced in humans and support the assertion that oral tolerance can be useful therapeutically.

4. A NIH clinical trial has been recently concluded which involved orally administering insulin to children at high risk for developing Type I diabetes (Diabetes Prevention Trial-1, DPT-1). Unfortunately, detailed results of this study have not yet been published. However a summary of the results in a subset of children with the highest levels of anti-islet cell antibodies that received oral insulin has been personally communicated to me by Dr. Noel MacLaren, an international expert in autoimmune

diabetes and participating investigator in the DPT-1 trial. Dr. MacLaren is currently a Professor of Pediatrics in the Department of Pediatrics at the Weill-Cornell Medical School. Dr. MacLaren was formerly at the University of Florida, College of Medicine. The oral administration of insulin significantly reduced the development of diabetes in the 5 years of the study in the subset of children with the highest levels of antibody to insulin. This study demonstrates the benefit to those at the highest risk for the disease. Additionally, a publication by MacLaren et al (New York Academy of Science, in press, 2005) demonstrates oral insulin also has a beneficial effect through oral tolerance in patients with established Type I diabetes.

Again, the results of these studies further demonstrate that oral tolerance can be induced in humans.

5. The Examiner has stated in the Office Action that the previous declarations that I have executed and were previously submitted in response to the Office Action mailed on February 10, 2004, admit that the experimental evidence provided therein was "unexpected" as thus seen as "an admission of the unexpected nature of the instant inventions". This is not the case. The use of the term "unexpected" in those declarations was merely to state that until the development of the present invention, one of skill in the art did not contemplate and thus would have not expected that an antigen could be successfully produced and administered in the same plant tissue which could then be orally administered. The presently claimed invention provides such a method. Accordingly, the presently claimed invention provides for

"unexpected" advantages over the prior art that were not previously realized; such unexpected effects were due to the fact that plant-derived antigens could be expressed in plants and administered orally such that the antigens are not adversely affected by digestion and thus can be more effective *in vivo*.

Thus, the plant could be used as both the expression vehicle and mode of administration for the antigen and this was never before contemplated.

6. The Examiner's assertion that the results provided in my two earlier executed declarations were just as or (more) likely due to "the specific animal model; the specific disease model; the specific antigen; or the specific transgenic plant species expressing the antigen" is unfounded. The experimental set up described in my two earlier declarations demonstrate favorable and beneficial results in mice. The experimental set-up is a credible *in vivo* model that is often used by those of skill in the art. The results provided in my earlier declarations clearly support the instant claims.

7. The Examiner has asserted that the animal data of record is not sufficient to satisfy the enablement requirement. However, as the Examiner stated during the above-noted interview, human *in vivo* data is not in fact required for enablement under United States law.

The animal data which has been provided in the present application would clearly be understood by those of skill in the art to support the use of the claimed methods and compositions in humans. In the case of both mice and humans, immune responses in


lymphocytes upon *in vitro* challenge to a specific protein is similarly attenuated or changed following oral administration of the protein. No qualitative or quantitative differences are found in the pattern of cytokines released or T cell activity and so mice and humans share a common biological response to oral protein antigens. Importantly, the NIH data in diabetes represents the clearest link yet between an autoimmune mouse disease model and autoimmune human disease, as both respond to an endogenous autoantigen protein that causes spontaneous diabetes.

8. The Examiner has rejected claims 52, 59-61, 63, 69-91 and 95 under 35 U.S.C. 103(a) as being unpatentable over WO 92/07581 in view of U.S. Patent No. 5,484,719 ("the '719 Patent"). See OFFICE ACTION at 3-4. WO 92/07581 discloses a method of suppressing an immune response by administering cell extracts from a donor. This reference does not teach any type of oral administration involving the use of any plant. The '719 Patent discloses expressing viral, bacterial or fungal antigens in a plant as a method of vaccination against harmful pathogens. This invention would only be useful for vaccination against pathogens which is a completely different use and thus method to that presently claimed. The presently claimed invention is not directed to vaccination to prevent any type of infectious agent. Thus, the '719 patent is not relevant to the presently claimed invention. Vaccination to pathogens using oral administration of pathogen antigens represents completely different mechanisms for beneficial effect to that of immune tolerance to the endogenous proteins involved in autoimmunity. One of skill in the art would not be motivated to combine the teachings of

these two references and, even if combined, it does not permit one skilled in the art to arrive at the methods claimed in the subject patent application.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: February 8, 2005



Anthony M. Jevnikar

EXHIBIT A

Oral Tolerance in Humans

T Cell but Not B Cell Tolerance After Antigen Feeding¹

Steffen Husby,^{2*} Jiri Mestecky,[†] Zina Moldoveanu,[†] Stephen Holland,^{*} and Charles O. Elson^{3*}

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The purpose of this study was to investigate whether oral tolerance, defined as Ag-specific immunologic unresponsiveness after Ag feeding, could be induced in humans after prolonged Ag ingestion. Eight adult volunteers ingested a total dose of 0.5 g of keyhole limpet hemocyanin (KLH) followed by subcutaneous immunization with KLH. Eight controls received only the subcutaneous immunization. In the group fed KLH, there was a significant reduction in KLH-specific T cell proliferation ($p = 0.04$) and delayed skin test responses ($p = 0.07$) to KLH. KLH ingestion alone did not induce significant levels of Abs in either serum or secretions. However, after the subsequent subcutaneous immunization, the number of circulating IgG and IgM anti-KLH-producing cells, the titers of serum IgG, IgA, and IgM anti-KLH Abs, and the titers of IgA anti-KLH Abs in saliva and intestinal secretions were significantly greater in the KLH-fed group than in the nonfed group. We conclude that KLH feeding induced systemic T cell tolerance, but B cell priming, at both systemic and mucosal sites. These studies support the concept of using Ag feeding as a treatment for certain immune-mediated diseases. *Journal of Immunology*, 1994, 152: 4663.

The environmental Ags from food and microbial flora are in constant contact with mucosal surfaces and provide a continuous stimulus for the entire immune system. Although a common result of such stimulation is the induction of mucosal and systemic immunity, an alternative outcome is a state of unresponsiveness or tolerance (1, 2). The term oral tolerance refers to a state of systemic unresponsiveness to parenteral immunization that is induced by previous Ag feeding. Oral tolerance of both humoral and cellular immunity has been convincingly demonstrated in rodents fed a wide variety of Ag types (3–8). In several experimental autoimmune diseases, such as experimental allergic encephalomyelitis (9–12), collagen-induced arthritis (13, 14), and experimental autoimmune uveitis (15), autoantigen feeding has blocked

induction of or ameliorated established disease. In some of these models, such Ag feeding has been found to induce CD8⁺ T cells that secrete the cytokine TGF- β upon Ag reexposure in vivo (16, 17). These results have prompted an interest in the feeding of autoantigens as a therapy for human autoimmune diseases (18, 19). However, attempts to induce oral tolerance in some species, such as rabbits, have been unsuccessful (20), and it is unclear whether this approach could be effective in humans, in whom the existence of oral tolerance has not been clearly demonstrated, although suggested (21, 22).

The purpose of this study was to determine whether Ag feeding of humans induces oral tolerance of either the T cell or B cell compartment. We used KLH,⁴ a potent systemic immunogen, because it is a novel Ag to most individuals, and it has been used safely in humans to assess immunocompetence. The strategy was to feed KLH to a group of volunteers; this group and another group not fed KLH were then parenterally immunized, and the ensuing systemic and mucosal immune responses were compared. T cell responses were assessed by a proliferation assay and by skin test reactivity to purified KLH. B cell responses were assessed by ELISA for IgM, IgG, and IgA anti-KLH

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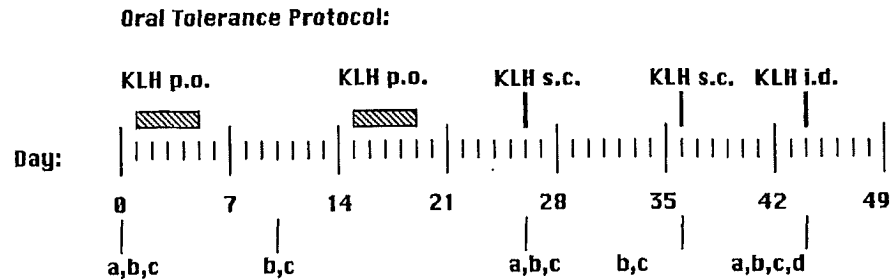
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⁴ Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; ELISPOT, enzyme-linked immunospot; SI, stimulation index; NC, nitrocellulose; SFC, spot-forming cell; EU, endotoxin units.

FIGURE 1. Protocol of feeding, parenteral immunization, and testing used in this project. KLH p.o. = 50 mg of KLH was administered in gelatin capsules per os daily; KLH s.c. = 100 μ g of KLH injected s.c.; KLH i.d. = 10 μ g of KLH injected intradermally as a skin test. Samples for testing were obtained as shown: a, peripheral blood cells for ELISPOT and proliferation assays; b, serum for ELISA; c, intestinal secretions and saliva for ELISA; d, delayed skin test responses.



Abs in serum, for IgA anti-KLH Abs in secretions, and by the ELISPOT technique for Ab-producing cells in peripheral blood.

Materials and Methods

Volunteers

A total of 16 healthy volunteers were recruited for the study; 8 of the volunteers (mean age 28 yr, range 23–37; 6 males and 2 females) took part in the study as the experimental group. The other 8 subjects were included in the control group (mean age 27 yr, range 21–36; 7 males and 1 female). The study was approved by the Human Use Committee for the University of Alabama at Birmingham. Informed consent was obtained from each subject before participation.

Experimental design

Fasting volunteers of the experimental group ingested 50 mg of KLH in gelatin capsules on days 1 to 5 and days 15 to 19. They were then immunized s.c. with 100 μ g KLH on day 25 and boosted with the same dose on day 36. The control group underwent the parenteral immunization only (Fig. 1). Samples of blood, saliva, and intestinal secretions were obtained at intervals for assessment of immunity. Blood samples were taken by venipuncture before the start (day 0) and on days 10, 25, 36, and 44 of the study. Secretions were obtained before (day 0) and on days 25 and 44 of the study. An intradermal skin test with KLH (10 μ g) was applied on day 44 and read on days 45 and 46.

Keyhole limpet hemocyanin

KLH as a freeze-dried powder was purchased from Sigma Chemical Co. (St. Louis, MO). For oral use, 50 mg of this preparation was packed into gelatin capsules, which were filled with lactose. For parenteral use, this preparation was dissolved in pyrogen-free saline and passed two times through a polymyxin-agarose column (Boehringer-Mannheim, Mannheim, Germany) at a concentration of 4 mg/ml. This treatment diminished the endotoxin content of the KLH preparation from approximately 1100 EU/ml to below 10 EU/ml as confirmed by Limulus assay (Whittaker, Walkersville, MD). The preparation was filter-sterilized, aliquoted in pyrogen-free saline plus 0.001% merthiolate, and stored at 4°C until use.

Cell isolation and purification

Heparinized blood was diluted 1:2 into Dulbecco's PBS (GIBCO BRL, Grand Island, NY), and the mononuclear cells were isolated by gradient centrifugation over Ficoll-sodium diatrizoate (Organon Teknica, Durham, NC) in 15 ml tubes at 2000 rpm for 20 min, the serum-Ficoll interface was collected and the cells were washed in Dulbecco's PBS followed by RPMI 1640 (GIBCO BRL), supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA), 100 U penicillin/ml and 100 μ g streptomycin/ml. The cells were

counted, adjusted to 5×10^6 /ml, and used directly in the ELISPOT assay. A T cell-enriched fraction was prepared by rosetting with 2-amino-ethylisothionium bromide-treated SRBCs, prepared as described (23) except that the rosetting was allowed to take place overnight on ice. Cells at the plasma-Ficoll interface were removed (E^- cells) and the T cell-enriched fraction (E^+ cells) was prepared by lysis of the SRBCs (24). The E^+ fraction typically contained 60 to 80% of the total number of cells. The E^- cells contained 40 to 50% monocytes as measured by the esterase stain for monocytes/macrophages (25). E^- cells were irradiated at 3000 rad before use as APCs in culture.

T cell proliferation assay

Sterile 96-well microtiter plates (Costar, Cambridge, MA) were used for cell culture. Quadruplicate wells were prepared with adherent APCs by the incubation of 100 μ l of 10^5 E^- cells/ml for 2 h. The wells were washed once with medium and the T cell-enriched (E^+) cells were added at 2×10^5 cells/ml. Replicate wells received KLH (10 μ g/ml), PHA (Sigma Chemical Co., 2 μ g/ml) as a positive control, or medium alone as a negative control. The plates were incubated at 37°C and 5% CO_2 for 2 days for PHA responses and 5 days for KLH-specific responses. The wells were then pulsed with [3H]thymidine (Amersham, Arlington Heights, IL) at 0.5 μ Ci/well for 6 h and harvested on nylon filters; cpm were measured with a liquid scintillation counter. The results were expressed both as a stimulation index (SI), i.e., the ratio of the mean KLH-stimulated cpm divided by the mean unstimulated cpm and as Δ cpm, i.e., the mean KLH-stimulated cpm minus the mean unstimulated cpm.

KLH-stimulated T cell cytokine production

T cells and APCs were cultured with KLH (10 μ g/ml) in wells of 24-well culture plates at 2×10^6 cells/well. After incubation at 37°C and 5% CO_2 for 48 h, the supernatants were harvested and frozen at $-20^\circ C$ until assay. The IFN- γ assay was performed using the WEHI 279 cell line, whose growth is inhibited by IFN- γ (26). TGF- β was measured in a bioassay using the cell line CH-1CAB, whose growth is inhibited by TGF- β (27). A standard curve was constructed for each cytokine and the values for the experimental samples interpolated. In both assays, cell growth was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide method as described (25).

Delayed skin test reactivity to KLH

Endotoxin-free KLH (10 μ g in 100 ml) was injected intradermally at the flexor surface of the forearm using pyrogen-free saline as the control. Induration was measured in mm at 24 h and 48 h by the same observer. A reaction was considered positive if any measurable induration was present.

Enumeration of Ab (spot)-forming cells (SFCs) by ELISPOT

To determine the numbers of IgG, IgA, and IgM anti-KLH-producing cells, the ELISPOT assay was used as described previously (29). Nitrocellulose (NC) 96-well microtiter plates (Millipore Corp., Bedford, MA) were incubated with KLH at a concentration of 20 $\mu\text{g/ml}$ in PBS overnight at room temperature. For the enumeration of isotype-specific Ig-producing cells, the wells were incubated with F(ab')_2 fragments of anti-IgG, anti-IgM, or anti-IgA Abs (Jackson ImmunoResearch Labs, West Grove, PA). Nonspecific protein binding to the NC was blocked by incubation for 2 h with PBS + 10% FCS. The mononuclear cell fraction from the heparinized blood sample was isolated as described above. 100 μl of cell suspension was immediately added in duplicate onto the plate at a range of concentrations from 10^5 to 5×10^6 cells/ml for anti-KLH Ab-producing cells and from 10^4 to 10^6 cells/ml for total Ig-producing cells. After incubation at 37°C for 3 h, the wells were washed three times with PBS and three times with PBS containing 0.05% Tween (Sigma Chemical Co.), and incubated overnight at 4°C with 100 μl of biotin-labeled goat F(ab')_2 anti-IgG, -IgA, and -IgM (Tago, Burlingame, CA) diluted 1:750 in PBS-Tween + 1% FCS. The wells were blotted dry, washed, and developed with Extravidin-alkaline phosphatase (Sigma Chemical Co.) followed by the chromogen substrate. The substrate was prepared by mixing 15 mg 5-bromo-4-chloro-3-indolylphosphate toluidine salt (Bio-Rad Labs, Richmond, CA) and 30 mg p-nitroblue tetrazolium chloride (Bio-Rad Labs), separately dissolved in 1 ml dimethylformamide, and adding the mixture to 100 ml 0.1 M NaHCO_3 + 1 mM MgCl_2 , pH 9.8. The NC plate was blotted dry and the wells were exposed to the chromogen substrate; blue spots appeared, usually within 30 min, where a positive reaction had occurred. When the spots had reached maximal intensity, the plate was rinsed with tap water and allowed to dry. The spots were enumerated under a stereomicroscope at 40-fold magnification. The active synthesis of Ab was confirmed by the incubation of the cells with cycloheximide (25 mg/ml for 2 h), which resulted in 70% to 100% inhibition of SFCs.

Intestinal and salivary secretions

Intestinal secretions and saliva samples were obtained from eight subjects in the fed group and from four subjects in the control group. The intestinal secretion samples were obtained with the use of a polyethylene glycol salt solution Colyte® (Reed & Carnrick, Piscataway, NJ), as described previously (30). Saliva was collected as unstimulated whole saliva by having the subject drool into a centrifuge tube placed in ice. Unstimulated parotid saliva was collected using a Schaefer cup placed over the parotid duct (31). The saliva samples were centrifuged at 10,000 rpm for 3 min in a microfuge to remove debris.

Measurement of Ab by ELISA

Flat bottom polystyrene microtiter plates (Titertek, Flow Labs., McLean, VA) were coated with KLH (10 $\mu\text{g/ml}$ of PBS), blocked with 5% FCS in PBS for 2 h, and washed three times with PBS-Tween. For analysis of serum samples, serum was diluted 1:250 and 1:1000 in PBS-Tween + 1% FCS. For analysis of saliva, the samples were diluted 1:10 and 1:50 in PBS-Tween containing 1% FCS. The plates were incubated overnight at room temperature. For analysis of intestinal secretions, the samples were diluted 1:4 and 1:40 in PBS-Tween + 5% FCS and incubated overnight at 4°C . The washed plates were incubated with biotinylated goat F(ab')_2 anti-IgG (1:8000), anti-IgA (1:1000), or anti-IgM (1:1000) (Tago) for 4 h at 37°C , followed by Extravidin-alkaline phosphatase (1:2000) for 2 h and then developed with para-nitrophenylphosphate substrate (Sigma Chemical Co.). The absorbance was read at 405 nm in a V_{max} kinetic microplate reader (Molecular Devices, Palo Alto, CA). A high titer serum was used as a reference standard and defined to contain 1000 U/ml IgG, 100 U/ml IgA, and 100 U/ml IgM anti-KLH Ab, based on their relative titer in serum. The absorbances of the samples were analyzed and converted into ELISA U/ml by using a computer program based on a four-parameter logistic model. Two control sera were included in each plate; based on the results of these control sera, the intra-assay variation was 3 to 7%, and the interassay variation of anti-KLH Abs was 22% to 26% for IgG, 15% to 26% for IgA, and 14% to 16% for IgM ($n = 26$).

Samples of sera from days 0, 25, and 44 were also tested for Ab to tetanus toxoid and bovine gammaglobulin. Serially diluted sera were tested in Ag-specific ELISA and the Ab concentrations were expressed as ng/ml by referring to calibration curves for each Ig (Ig) isotype, constructed by assaying wells on the same plates coated with anti-Ig isotype Abs and calibrated serum standards.

Total Ig levels in secretions

The amount of IgG, IgA, and IgM in intestinal secretions was assessed by ELISA. Microtiter plates (Titertek, Flow Labs) were coated with affinity-purified goat F(ab')_2 anti-IgG (2.5 $\mu\text{g/ml}$), anti-IgA (5 $\mu\text{g/ml}$), or anti-IgM (2.5 $\mu\text{g/ml}$) in PBS (all from Jackson ImmunoResearch Labs), blocked with 5% FCS in PBS, and washed three times. The samples were added at a dilution of 1:400 and 1:4000 in PBS-Tween + 5% FCS and incubated overnight at 4°C . The reference standards used were purified colostral IgA (2.9 g/l, Ref. 32) and the Monitrol standard (Baxter, McGaw Park, IL) for IgG (9.9 g/l) and for IgM (0.9 g/l). The plates were washed and incubated with biotin-labeled F(ab')_2 anti-IgG, anti-IgA, or anti-IgM (Tago), and developed as above for the Ab determinations.

Statistical analysis

Nonparametric statistical analysis was used. Comparison between groups was performed with the Mann-Whitney U-test for unpaired samples. Comparison within groups was done with the Wilcoxon/Pratt test for comparing two samples and the Friedman test for comparing several samples. The level of significance was chosen as $p < 0.05$.

Results

Induction of T cell tolerance by feeding

T cell proliferation assay. Ag-specific T cell proliferation was assessed before immunization (day 0), after the oral immunization (day 25), and after the parenteral immunization (day 44) (Fig. 2). The SI in both groups was low before the immunization. After the oral immunization, the SI for the KLH-fed group rose moderately but significantly ($p = 0.047$) and at this time point was also higher than the preimmunization value of the control group ($p = 0.007$). However, after the parenteral immunization, the SI in the KLH-fed group was significantly lower than in the nonfed group ($p = 0.04$) (Fig. 2A). Similar results were found when the data are expressed as Δ cpm, with an increase after the feeding in five of eight subjects, followed by a reduction to baseline levels after the parenteral immunization (Fig. 2B). Mean PHA-stimulated T cell proliferation of the KLH-fed group did not change significantly in the KLH-fed vs control groups either after the feeding or after the parenteral immunization, nor in the KLH-fed group before feeding, after feeding, or after parenteral immunization (data not shown).

Delayed skin test response. Intradermal skin testing with KLH was done on day 44 (Table I). At 24 h, only one of eight subjects in the KLH-fed group had a positive reaction, whereas seven of eight in the control group were positive ($p = 0.007$). The same pattern was seen at 48 h, when zero of eight in the fed group were positive vs five of eight in the control group ($p = 0.038$).

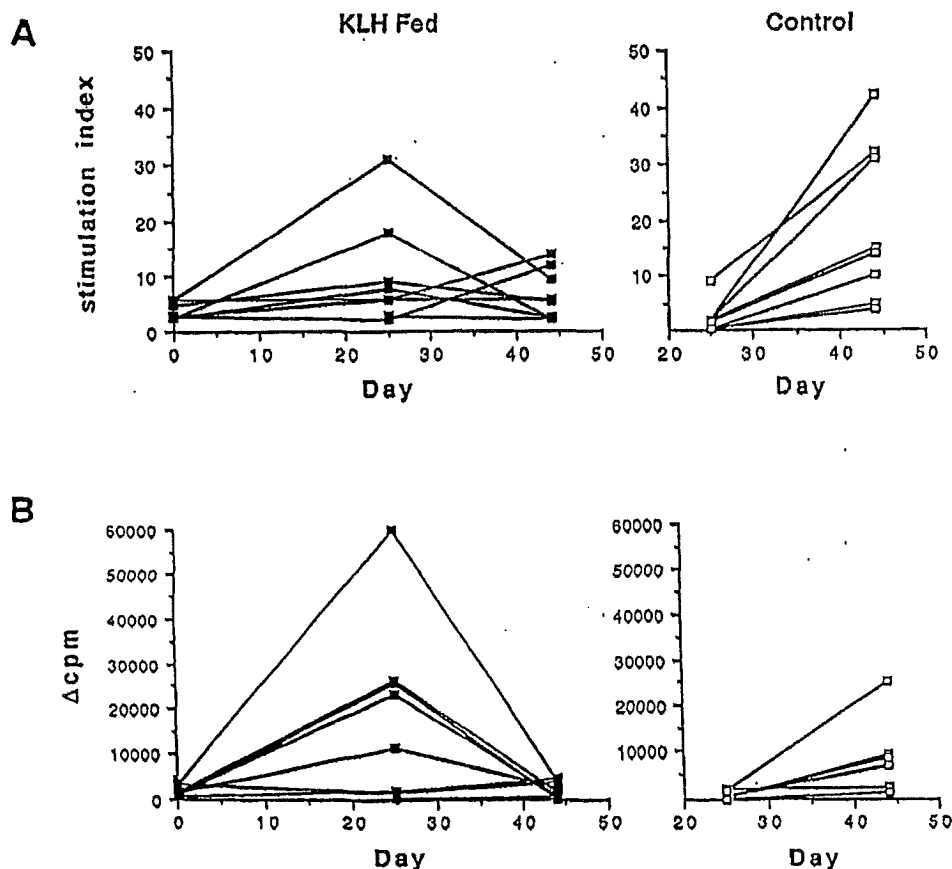


FIGURE 2. KLH-induced proliferation of peripheral blood T cells. The data from each individual shown at day 0 before the study, at day 25 after the oral feeding, and at day 44 after subcutaneous immunization. A, stimulation index; B, Δ cpm.

Priming of the systemic B cell response by KLH feeding

Anti-KLH SFCs in peripheral blood. Virtually no anti-KLH SFCs were identified in the blood either before or after the KLH feeding. However, IgG, IgA, and IgM anti-KLH SFCs were detected after the parenteral priming and booster doses (Fig. 3). IgG and IgM anti-KLH-secreting cells were significantly greater in number in the KLH-fed group than in the control group only after the parenteral priming on day 36 ($p = 0.028$ and $p = 0.021$, respectively). There were no statistically significant differences on day 44.

Serum anti-KLH Abs. Feeding with KLH did not produce any detectable serum levels of anti-KLH Abs of any isotype above the background level (Fig. 4). However, KLH feeding did result in the priming of B cells, as demonstrated by a significantly higher serum IgG anti-KLH (Fig. 4A) in the KLH-fed group as compared with controls following the parenteral priming and booster immunization ($p < 0.05$). Both IgA and IgM anti-KLH in the KLH-fed group rose sharply and significantly ($p < 0.01$ for both; Figs. 4, B and C) after the parenteral priming, but were not significantly different from the control group after the parenteral booster.

Serum Abs against a food Ag, bovine gammaglobulin, as well as against tetanus toxoid, were measured in samples obtained at days 0, 25, and 44. No change in mean Ab titer in either the KLH-fed or the control group was found for either of these Ags (data not shown).

Priming of secretory Abs by KLH feeding

Anti-KLH Ab levels in salivary secretions. The IgA anti-KLH levels in both whole saliva (Fig. 5A) and parotid saliva (data not shown) of the KLH-fed group did not increase significantly after the feeding alone, but did increase after the subcutaneous immunization with KLH (day 44) ($p < 0.005$ for whole saliva and $p < 0.015$ for parotid saliva).

Anti-KLH Ab levels in intestinal secretions. No IgG or IgM anti-KLH was detected in the intestinal secretions. IgA anti-KLH levels in intestinal secretions were low, and although they were enhanced after the KLH feeding, this increase was not statistically significant. However, secretory IgA anti-KLH was increased in the KLH-fed group after the parenteral immunization (Fig. 5B) when compared with their own day 0 base line levels ($p = 0.016$).

KLH-stimulated cytokine secretion. On days 0, 25, and 44, T cells were cultured with APC and KLH (10 μ g/ml) for

Table 1. Delayed skin test response to KLH^a

Group	24 h		48 h	
	Mean (range)	Number of positive/total	Mean (range)	Number of positive/total
KLH-fed	1.2 (0–10)	1/8	0 (0–0)	0/8
Controls	11.9 (0–23)	7/8	6.6 (0–20)	5/8
<i>p</i> -value	0.007		0.038	

^a KLH (10 µg) was injected intracutaneously and the reaction measured as induration (mm) at 24 and 48 h after the injection.

48 h. Supernatants of those cultures were collected and tested for IFN- γ and TGF- β by bioassay. IFN- γ was not detected in any supernatant. TGF- β was detected in a small number of culture supernatants, but there was no relationship to KLH feeding or immunization (data not shown).

Discussion

The present study demonstrates that oral tolerance can be induced in humans; however, with the Ag, dosage, and immunization schedule used, tolerance was limited to the T cell compartment, as demonstrated by a reduction in Ag-specific T cell proliferation and markedly diminished delayed skin test reactivity. Although no anti-KLH-secreting cells or Abs were detected in blood or secretions after the oral feeding, the KLH feeding clearly had an impact on the immune system in that it primed B cells in systemic as well as secretory sites for a greater response upon parenteral immunization. Because the dose, frequency, and type of Ag are known to have a profound influence on the induction of oral tolerance in experimental animals (7, 8), it is possible that varying one or more of these parameters might induce tolerance in B cells in humans as well.

The purpose of this study was to determine whether oral tolerance to protein Ag existed in humans; a detailed examination of the Ag specificity of the tolerance was not planned as a part of the study because it is well established in experimental animals that oral tolerance to proteins is Ag-specific. However, the absence of any effect of KLH feeding on polyclonal T cell proliferation and on serum Ab titers to two common Ags does suggest that oral tolerance is Ag specific in humans as well.

Tolerance of T cells but not B cells has been previously identified after feeding (33, 34) and injecting (35–37) low doses of Ag to animals. A recent study performed in transgenic mice showed that the small amounts of autoantigen released spontaneously in vivo rendered the animals' T cells but not B cells tolerant (38). The B cell system can be tolerized by Ag feeding, but generally requires larger amounts of Ag (4–6). Sensitivity to tolerance induction also varies among T cell subsets. CD4⁺ T cells can be divided into two major subgroups based on their production of cytokines. CD4⁺ Th1 cells produce IL-2 and IFN- γ and mediate delayed hypersensitivity. CD4⁺ Th2 cells produce IL-4, IL-5, IL-6, IL-10 and provide help for B cell

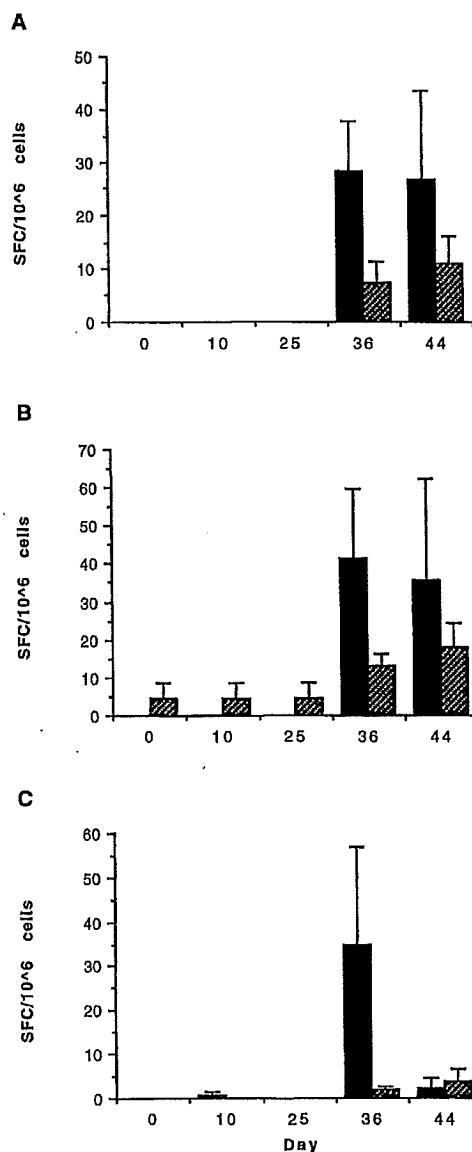


FIGURE 3. Circulating anti-KLH secreting cells (SFC)/10⁶ PBLs of the (A) IgG, (B) IgA, and (C) IgM isotype before and after oral feeding and parenteral immunization with KLH. Solid bars denote the KLH-fed group and lined bars the control group. The data for the control group at days 0, 10, and 25 represent the preimmunization value. Data are shown as geometric means and SE.

Ab responses (39). Parenteral injection of soluble protein Ags into mice tolerized Th1 but not Th2 cells (40); the hyporesponsiveness in Th1 cells was mediated by IL-4 production by Th2 cells (41). There seems to be a gradient of sensitivity to tolerance induction, with Th1 cells > Th2 cells > B cells. Although the existence of the Th1/Th2 subsets is not as well established in humans as compared with mice, the present data are consistent with this paradigm. KLH-specific T cell proliferation and skin test responses are mainly Th1 functions, and these were significantly inhibited by KLH feeding. The B cell priming that

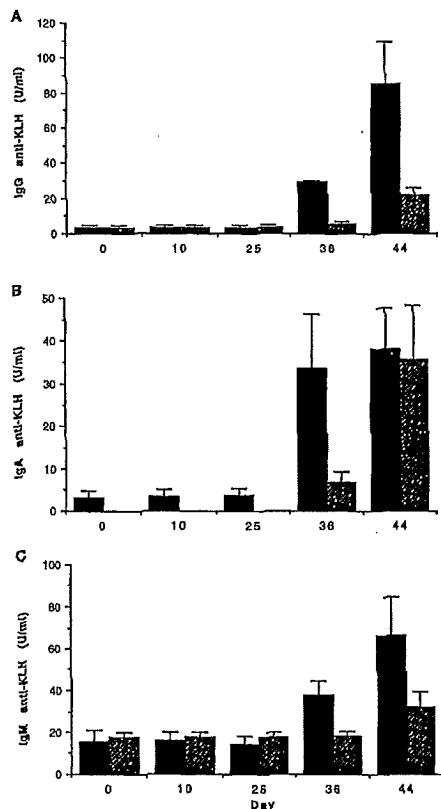


FIGURE 4. Serum Abs to KLH. Anti-KLH of (A) the IgG, (B) the IgA, and (C) the IgM isotype, obtained before the start of the study (day 0), after one period of KLH feeding (day 10), after two periods of KLH feeding (day 25), after parenteral priming (day 36) and after parenteral booster (day 44). The KLH-fed group is denoted by solid bars and the control group by lined bars. For the control group the results presented at days 0, 10, and 25 represent the preimmunization value. Bars represent geometric means and SE.

occurred from the KLH feeding is consistent with a lesser effect of the feeding on Th2 helper cells and B cells. The lack of any Ab production from the feeding alone could result from the dose of Ag being insufficient to trigger Th2 cells or KLH feeding having some inhibitory effect on Th2 cell function.

Tolerance may occur by a number of mechanisms, including clonal deletion, clonal anergy, or suppression. With regard to orally induced tolerance, local and systemic suppressor T cell circuits may be particularly important (5, 9, 42–44). Antigen feeding can generate Ag-specific suppressor T cells in the Peyer's patches of mice, and these T cells later populate systemic lymphoid tissues such as the spleen (42). However, even after suppressor T cells can no longer be identified, the animals remain unresponsive to the fed Ag (43), suggesting the presence of an additional mechanism such as clonal anergy. Ag feeding has been reported to stimulate CD8⁺ T cells that release the immune-inhibitory cytokine TGF- β upon restimulation with

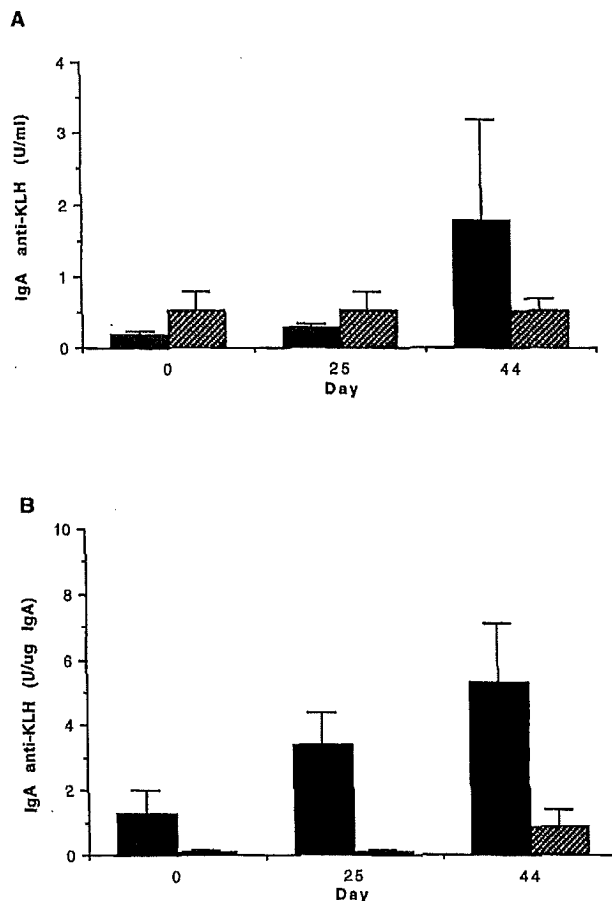


FIGURE 5. IgA anti-KLH Abs in secretions: (A) in whole saliva and (B) in intestinal secretions. Solid bars denote KLH-fed group and lined bars denote control group (four subjects only). For the control group, the results from days 0 and 25 represent the preimmunization value. Data are shown as geometric means and SE.

Ag (16, 17). Accordingly, peripheral blood T cells from KLH-fed and control subjects were restimulated with KLH plus APCs *in vitro*. TGF- β was detected in a small number of samples, but its presence was inconsistent and had no relationship to either the feeding or parenteral immunization with KLH. These data do not exclude a possible role for TGF- β -producing cells, in that the relevant cells may not circulate in sufficient numbers to be detected. Studies detecting them have used lymphoid and other tissues (16, 17), not circulating cells. The role of this or other cytokines, such as IL-4, in oral tolerance remains to be defined.

Although the cellular and molecular mechanisms for the induction of oral tolerance in humans are unknown, oral tolerance may represent an important immunoregulatory process that limits immune response to innocuous food Ags. Certainly, humans ingest food Ags daily in quantities that should result in tolerance, and a small fraction is known to be absorbed into the circulation (45). When

adults with low levels of Ab to BSA were immunized by either ingestion or parenteral injection of BSA, they did not develop an Ab response (21), which may represent a form of oral tolerance to this food Ag. Despite the apparent occurrence of tolerance to food Ags, secretory and serum Abs to them are readily detectable in humans (46–48). The gradient of sensitivity of T cell subsets and B cells discussed above may explain this apparent paradox. Prolonged ingestion of Ag may be sufficient to prime B cells and Th2 cells sufficiently for the production of low levels of Ab.

Despite the large amount of secretory IgA Ab produced daily, it has been difficult to induce secretory IgA responses at mucosal surfaces in any species, including humans, by oral immunization with soluble or nonviable particulate Ags (49). The present results illustrate this, in that even 10 days of Ag feeding did not result in significant Ab production in saliva or intestinal secretions. Thus, these results have implications relative to strategies for oral vaccines. One prediction is that oral-parenteral combinations may be more effective for immunization in humans than the oral route alone. Another possibility is that protein Ags given orally will require mucosal adjuvants to prevent induction of T cell tolerance and to effectively activate both T cells and B cells (49).

These results support the idea that it may be possible to exploit orally induced tolerance in the treatment of human disease such as allergy or autoimmunity. Preexisting IgE responses and delayed hypersensitivity have been successfully down-regulated by Ag feeding in experimental animals, thus providing experimental support to this notion (50, 51). In regard to allergy, hyporesponsiveness to allergens such as tree or grass pollens and leaf extracts has been induced with variable success (52, 53). The induction of T cell tolerance to ingested Ags may be of considerable importance in the amelioration of diseases in which T cells represent the dominant effector mechanism. As mentioned above, oral tolerance to ingested autoantigens has been effectively used in experimental autoimmune diseases (7–13). Whether the feeding of autoantigens to humans can suppress the further progression of autoimmune disease remains to be demonstrated, but clinical studies are under way in multiple sclerosis (54) and rheumatoid arthritis (55). If this can be achieved, the induction of T cell tolerance by Ag feeding may represent a novel form of treatment for autoimmune diseases and hypersensitivity disorders.

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EXHIBIT B

INDUCTION OF IMMUNE TOLERANCE TO HUMAN TYPE I COLLAGEN IN PATIENTS WITH SYSTEMIC SCLEROSIS BY ORAL ADMINISTRATION OF BOVINE TYPE I COLLAGEN

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Objective. To determine whether oral tolerance to type I collagen (CI) could be induced in patients with systemic sclerosis (SSc).

Methods. Twenty adult patients with limited or diffuse SSc were enrolled in a study to receive 0.1 mg of solubilized native bovine CI daily for 1 month, followed by 0.5 mg daily for 11 months. Peripheral blood mononuclear cells (PBMC) were obtained from the patients and cultured with human $\alpha 1(I)$ and $\alpha 2(I)$ chains, before and after CI treatment. Culture supernatants were analyzed for levels of interferon- γ (IFN γ) and interleukin-10 (IL-10). Sera obtained before and after treatment were analyzed for levels of soluble IL-2 receptor (sIL-2R). Although this study was not intended to assess the clinical efficacy of oral CI administration in SSc, selected measures of disease severity and organ involvement were evaluated.

Results. Oral administration of CI to SSc patients induced significant reductions in levels of IFN γ and IL-10 in $\alpha 1(I)$ - and $\alpha 2(I)$ -stimulated PBMC culture supernatants, indicating that T cell immunity to CI was decreased by this treatment. Serum levels of sIL-2R also decreased significantly after oral CI treatment, suggesting a reduction in T cell activation. Significant improve-

ments occurred in the modified Rodnan skin thickness score and the modified Health Assessment Questionnaire after 12 months of oral CI in this open trial. The lung carbon monoxide diffusing capacity improved statistically and showed a trend toward clinically significant improvement.

Conclusion. Oral administration of bovine CI to patients with diffuse or limited SSc induces a reduction in T cell reactivity to human CI, appears to be well tolerated, and does not worsen the disease. Further evaluation of oral tolerance to CI in patients with SSc is justified to determine whether it has therapeutic efficacy.

Type I collagen (CI) is the most abundant of all collagens in humans (1). It is present in blood vessels, skin, lungs, heart, kidneys, and intestines, all of which are affected in systemic sclerosis (SSc) (1). CI is a heterotrimer molecule composed of two identical $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain (1). Each α chain contains 1,014 amino acid residues (1). Human and bovine CI have ~92% homology at the amino acid level (2,3). Evidence for cellular immunity to CI in SSc patients was first demonstrated by our group in 1976 (4) and has been confirmed by other investigators (5,6). We found that peripheral blood mononuclear cells (PBMC) from 92% of SSc patients produce chemotactic cytokines when cultured with CI, whereas only 8% of PBMC from healthy subjects do so (4). Hawrylko et al (5) also showed that peripheral blood CD4⁺ T cells from patients with SSc produce interleukin-2 (IL-2) in a dose-dependent manner in response to stimulation with human CI, while those from healthy subjects do not.

A major portion (approximately one-third) of the body's immune cells reside in the gut-associated lymphoid tissue (GALT) (7). The GALT is particularly effective in mounting a tolerogenic response to ingested soluble proteins (7,8). This process, called oral toler-

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ance, has been repeatedly demonstrated in laboratory animals. For example, when mouse strains susceptible to experimental allergic encephalomyelitis (EAE) after systemic immunization with myelin basic protein (MBP) are fed MBP prior to immunization, they develop less EAE or no EAE compared with placebo-fed MBP-immunized controls (9).

The mechanisms that mediate oral tolerance include active cellular suppression (regulatory T cells), clonal anergy, and clonal deletion (10–12). The particular dose of antigen and the frequency of feeding determine which mechanism(s) predominates (10,12). Multiple oral feedings of low-dose soluble antigen favor development of regulatory CD4⁺ T cells that secrete Th2 cytokines, such as IL-4 and IL-10, and transforming growth factor β 1 (TGF β 1)–secreting T cells (Th3 cells) (10,12). These regulatory T cells migrate to peripheral sites throughout the body, and when they encounter the antigen to which they are tolerized, they collectively secrete IL-4, IL-10, and TGF β 1, which can down-regulate Th1 CD4 cells reacting to a variety of antigens, a process called “bystander suppression” (10,13,14).

Since many of the antigens that are involved in human autoimmune diseases are unknown, it is theoretically possible that, by feeding low doses of antigen from the organs or tissues that are the target of autoimmune attack, T cell responses to other autoantigens perpetuating the disease can be down-regulated. CI qualifies as a candidate oral tolerance antigen in SSc, in that it is present in all of the target organs. Since most SSc patients exhibit sensitization to CI (1,4–6), as manifested by cytokine production by PBMC during culture with CI or constituent α 1 and α 2 chains, successful tolerization to CI after it has been orally administered to SSc patients can be assessed by determining whether there are decreases in cytokine production by PBMC cultured with CI α chains. The present phase I study was undertaken to determine whether daily administration of oral bovine CI to patients with SSc would result in down-regulation of the immune response to human CI.

PATIENTS AND METHODS

Patient recruitment and characteristics. This study was approved by the Institutional Review Board at The University of Tennessee Health Science Center. Patients were recruited from University of Tennessee and community rheumatology practices in Memphis. Inclusion criteria were as follows: age \geq 18 years; diagnosis of limited or diffuse SSc by the criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) (15); and patient's PBMC demonstrated reactivity to bovine CI, as

defined by the production of IL-10 (\geq 2 times baseline production) when cultured with native bovine CI. Patients taking D-penicillamine, captopril, or calcium channel blockers were required to be receiving stable doses of these agents for at least 3 months prior to enrollment. The maximum allowable dosage for D-penicillamine was 750 mg/day. Patients taking corticosteroids were required to be receiving a stable dose for at least 1 month prior to enrollment; the maximum allowable dosage was 10 mg/day of prednisone equivalent.

Patients were excluded from the study for the following reasons: inability to render an informed consent in accordance with institutional guidelines; receiving another investigational drug (excluding D-penicillamine) within 90 days of study initiation; a concurrent serious medical condition that, in the opinion of the investigators, made the patient inappropriate for the study; an SSc-like illness associated with environmental, ingested, or injected agents, such as L-tryptophan, tainted rapeseed oil, vinyl chloride, or bleomycin; morphea, linear scleroderma, or eosinophilic fasciitis; a positive pregnancy test; use in the previous 3 months of cyclophosphamide, cyclosporin A, methotrexate, or azathioprine; allergy to beef; or malabsorption syndrome.

Design and duration of the study. The study was an open-label trial to determine whether oral CI treatment would down-regulate PBMC cytokine production when cultured with α 1(I) and α 2(I). Patients received 0.1 mg/day of solubilized bovine CI for 1 month, followed by 0.5 mg/day for 11 months. Collagen was solubilized in 0.1M acetic acid and aliquoted into individual-dose vials. Patients kept the vials refrigerated. Each morning, the patient added 1 vial of the CI preparation to 4–6 ounces of cold orange juice and drank it just before eating breakfast. Patient compliance was monitored by counting the numbers of empty and full vials returned at each visit.

Concomitant medication. Patients were not allowed to increase dosages of D-penicillamine, captopril, calcium channel blockers, or corticosteroids during the study. Patients were dropped from the study if increases in any of these medications were deemed medically necessary by their primary physicians.

Clinical measurements. Significant clinical responses were not expected due to the small study size and the variability in disease classification, manifestations, and duration. However, the following measures of disease severity and organ involvement were evaluated: modified Rodnan skin thickness scores (MRSS) (16) at 0, 1, 2, 3, 6, 9, and 12 months; pulmonary function tests (PFTs; spirometry) with measurement of the diffusing capacity for carbon monoxide (DLCO; performed by the same personnel using the same equipment) at 0, 3, 6, 9, and 12 months; serum creatinine levels at 0, 1, 3, 6, 9, and 12 months; and the modified Health Assessment Questionnaire (M-HAQ) (17,18) at 0, 3, 6, 9 and 12 months.

Microculture of SSc PBMC with α 1(I) and α 2(I). Briefly, before and after 3, 6, and 12 months of oral bovine CI treatment, PBMC were isolated by Ficoll-Hypaque density-gradient centrifugation and set up in culture in 48-well tissue culture plates (2×10^6 cells in 0.5 ml of RPMI 1640 containing 100 units/ml of penicillin, 100 μ g/ml of streptomycin, 2 mM L-glutamine, 20 mM HEPES, and 5% fetal calf serum). Cultures were set up with 50 μ g/ml each of purified bovine α 1(I) and α 2(I) chains, and phytohemagglutinin (PHA; 10 μ g/ml) and phosphate buffered saline (PBS) as controls in duplicate wells. After 5 days of culture, supernatants from duplicate

wells were pooled, harvested by centrifugation, and frozen at -70°C until assayed for cytokine levels (within 30 days).

Measurement of cytokines in serum and PBMC supernatants. After screening for several cytokines in supernatants from SSc PBMC cultured with $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$, we found that most SSc patients produced increased amounts of interferon- γ ($\text{IFN}\gamma$) and IL-10 protein, as measured by enzyme-linked immunosorbent assay (ELISA). These cytokines were subsequently measured in all culture supernatants.

$\text{IFN}\gamma$ and IL-10 levels were measured by commercial ELISA (R&D Systems, Minneapolis, MN) in supernatants harvested from microcultures of SSc PBMC stimulated by PHA, $\alpha 1(\text{I})$, and $\alpha 2(\text{I})$, and PBMC plus PBS as a control for background cytokine production. A positive response to $\alpha 1(\text{I})$ or $\alpha 2(\text{I})$ was arbitrarily defined as IL-10 or $\text{IFN}\gamma$ levels in $\alpha 1(\text{I})$ - or $\alpha 2(\text{I})$ -stimulated PBMC culture supernatants that were ≥ 2 times the respective cytokine level in the PBMC plus PBS control supernatant. Soluble IL-2 receptor (sIL-2R) levels were measured by ELISA (R&D Systems) in sera obtained before and after 12 months of oral CI treatment. All samples were tested in duplicate.

Measurement of T cell subsets by flow cytometry. Isolated PBMC obtained from samples taken at 0 and 6 months of oral CI treatment were reacted with a panel of monoclonal antibodies that recognize T cell-specific markers, CD4+, CD8+, CD3+DR+, CD4+CD45+RA+, CD4+CD45+R0+, CD8+CD45+RA+, CD8+CD45+R0+, and CD4+CD26+ and analyzed by fluorescence-activated cell sorter at the University of Tennessee Molecular Resource Center.

Preparation and handling of bovine CI. Bovine CI was prepared as previously described (19). Bovine fetuses from pregnant cows were obtained from a local slaughterhouse within 1 hour of death. The skins of 4 fetal calves were removed and maintained at 4°C throughout the preparation. The tissue was sliced into strips and processed through a household meat grinder, then homogenized in a Waring blender with ice chips. The homogenate was centrifuged (10,000g) for 30 minutes and reextracted twice with 1M NaCl (pH 7.6, with 0.05M Tris HCl) and twice with 0.1M acetic acid to remove some type III soluble collagen and much of the noncollagenous components. The final pellet (~ 500 gm) was suspended in 16 liters of 0.1M acetic acid, and the pH was adjusted to 2.8 with formic acid.

Type I collagen was solubilized by overnight (16 hours) digestion with 20 gm of pepsin ($3\times$ crystallized; Sigma, St. Louis, MO) at 4°C . The digest was centrifuged (10,000g for 30 minutes), and the insoluble pellet was discarded. Type I collagen in the supernatant was precipitated by addition of 5M NaCl solution to a final concentration of 0.8M. This was centrifuged as before, and the pellet was redissolved in 0.1M acetic acid. The pH was adjusted to 7.4 with 0.05M Tris and 10M NaOH to inactivate pepsin. Solid NaCl was added to a concentration of 1M, and the solution was centrifuged. The supernatant was collected, and the NaCl content was increased to 1.7M with 5M NaCl. This was centrifuged to remove contaminating CIII. The 1.7M NaCl supernatant was further adjusted to 2.5M NaCl, which precipitated the CI.

The CI pellet was collected by centrifugation and redissolved in 0.5M NaCl, 0.05M Tris, diluted to 0.2M NaCl with water, and 50 gm of DE-52 was added to create a slurry.

This was stirred overnight and centrifuged to remove any DEAE that had bound any remaining pepsin and contaminating glycosaminoglycans. The supernatant was dialyzed against 0.02M NaH_2PO_4 to precipitate CI. The pellet was redissolved in 0.01M acetic acid, dialyzed exhaustively against the same, and stored at -80°C until used.

The homogeneity of the CI was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which showed an $\alpha 1(\text{I})/\alpha 2(\text{I})$ ratio of 2:1 with no contaminating type V or type III collagen. The $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$ collagen chains were separated by carboxymethyl cellulose chromatography, and constituent α chains were digested with cyanogen bromide (19).

Frozen CI stock containers were allowed to thaw over 2–3 days at 4°C prior to dispensing into vials. Thawed collagen was centrifuged at 4°C at 12,000g to remove particulates. The collagen was diluted to a concentration of 50 $\mu\text{g}/\text{ml}$ or 250 $\mu\text{g}/\text{ml}$ with cold (4°C) 0.1M acetic acid. The diluted CI was filtered at 4°C through a glass fiber Acrodisc (Gelman Sciences, Ann Arbor, MI) and then a 0.45 μ filter (Nalgene filter; Nalge, Rochester, NY) and aliquoted (2 ml) into sterile 2-ml screw-top polypropylene vials (Nalgene vials; Nalge). Vials were placed in plastic bags (35 vials/bag) and stored frozen at -20°C until given to the patients.

Statistical analysis. Cytokines produced by PBMC in response to culture with bovine CI, serum levels of sIL-2R, results of PFTs, and clinical variables were analyzed by Student's paired *t*-test to determine whether significant changes occurred after 3, 6, 9, or 12 months of oral CI treatment, compared with pretreatment values. Correlations of the M-HAQ or the MRSS versus IL-10 or $\text{IFN}\gamma$ were analyzed by Spearman's correlation test.

RESULTS

Patient characteristics at study entry. Twenty-five patients with SSc were screened. Twenty-four exhibited production of IL-10 or $\text{IFN}\gamma$ that was ≥ 2 -fold higher than the levels in parallel cultures of the patients' PBMC plus PBS but without $\alpha 1(\text{I})$ or $\alpha 2(\text{I})$. Four patients had complications of SSc or other medical diseases that disqualified them from the study. One patient was enrolled but withdrew from the study very early. The characteristics of the remaining 19 patients are shown in Table 1.

The patients were predominantly female, and the majority had late, diffuse disease. All patients satisfied the ACR preliminary criteria for the classification of SSc. Thirteen were white and 6 were African American. Only 3 patients had a disease duration of < 2 years. Only 3 patients were currently taking D-penicillamine. The 5 patients taking nonsteroidal antiinflammatory drugs (NSAIDs) discontinued these during the last 6 months of CI treatment. Three patients took 5 or 10 mg/day of prednisone throughout the study period.

Side effects, withdrawals, and compliance. Seventeen patients were treated for 12 months. Two patients dropped out because of difficulty with transportation: one

Table 1. Characteristics of the patients taking oral type I collagen for 1–12 months*

Sex	
Female	15
Male	4
Race	
White	13
African American	6
SSc type	
Diffuse SSc	14
Limited SSc	5
Age, mean \pm SD years	50.7 \pm 2.6
Disease duration	
Mean \pm SD years	9.1 \pm 2.0
<2 years' duration	3
Medication use	
Penicillamine	3
NSAIDs	5
Prednisone	3

* Except as noted otherwise, values are the number of patients. SSc = systemic sclerosis; NSAIDs = nonsteroidal antiinflammatory drugs.

very early (<1 month), the other after 6 months. One patient developed a foot drop of uncertain etiology and was removed from the study after 6 months of therapy. No other possible side effects were noted. There was 100% compliance by each patient until the time each dropped out of the study or the study was completed.

Induction of T cell tolerance to CI by administration of oral CI. The daily administration of bovine CI for 12 months was accompanied by significant reductions in IFN γ production by PBMC cultured with purified α 1(I) and α 2(I) chains of human CI as measured after 6 and 12 months of treatment (Figure 1A). IFN γ is a Th1 cytokine, and its reduced production by α 1(I)- and α 2(I)-stimulated PBMC suggests that oral tolerance to CI was effected. Quite surprisingly, IL-10 levels in the same PBMC culture supernatants were also significantly reduced after 3, 6, and 12 months of oral CI treatment (Figure 1B). The production of IFN γ and IL-10 by PBMC stimulated with PHA was not statistically different before or at 3, 6, or 12 months after oral CI treatment (results not shown).

After 12 months of oral CI treatment, there was a significant reduction (as determined by Student's paired *t*-test) in the serum levels of sIL-2R (Figure 2).

T cell subsets measured by the following markers did not change after treatment with oral CI treatment: CD8+, CD4+, CD3+DR+, CD4+CD45+RA+, CD4+CD45+RO+, CD4+CD26+, CD8+CD45+RA+, and CD8+CD45+RO+ (results not shown).

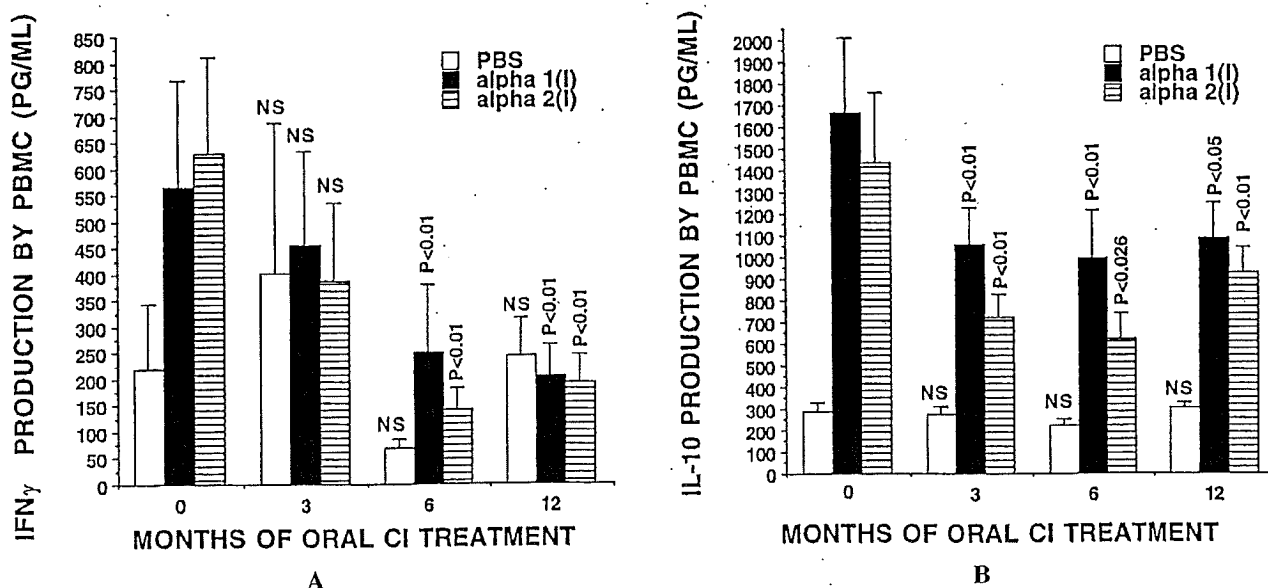


Figure 1. Production of A, interferon- γ (IFN γ) and B, interleukin-10 (IL-10) by peripheral blood mononuclear cells (PBMC) from patients with systemic sclerosis. PBMC were cultured with α 1(I) and α 2(I) chains before and after 3, 6, and 12 months of oral treatment with bovine type I collagen (CI; 500 μ g/day). Harvested culture supernatants were analyzed for levels of IFN γ and IL-10 by commercial enzyme-linked immunosorbent assay, as described in Patients and Methods. Values are the mean and SEM. *P* values determined by Student's paired *t*-test; NS = not significant.

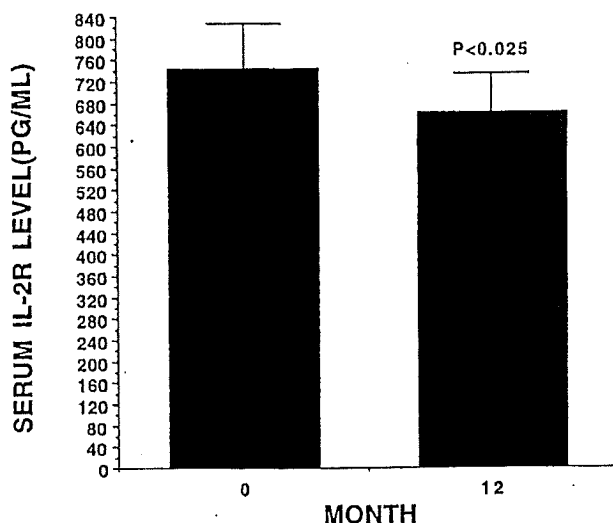
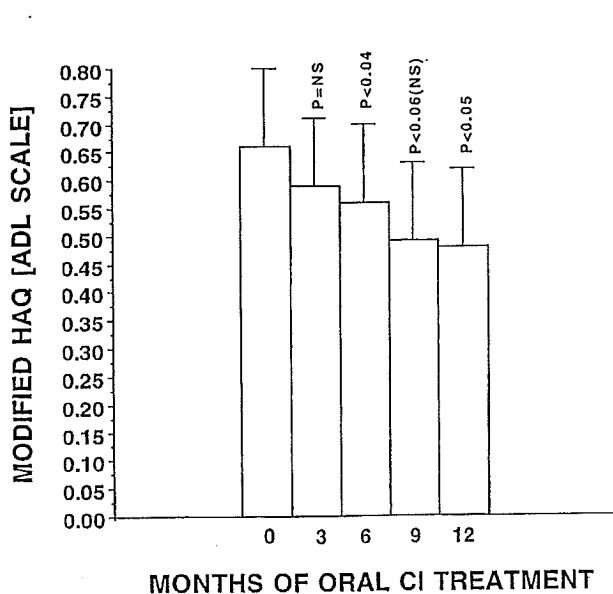


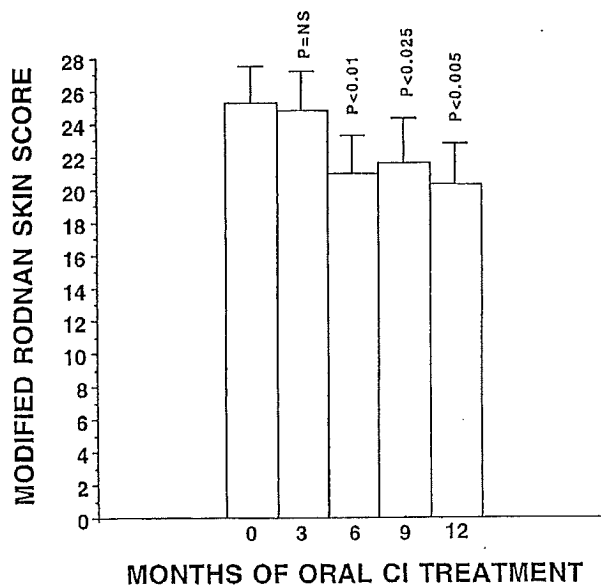
Figure 2. Serum levels of soluble interleukin-2 receptor (IL-2R) in patients with systemic sclerosis. Sera were obtained before and after 12 months of oral treatment with bovine type I collagen and analyzed by commercial enzyme-linked immunosorbent assay for soluble IL-2R levels. Values are the mean and SEM of the 17 patients completing 12 months of treatment. *P* value determined by Student's paired *t*-test.

Improvement in clinical variables. The M-HAQ difficulty in performing activities of daily living (ADL) scale and the MRSS were significantly improved after 6 and 12 months in this open-label study (Figures 3A and B). After 12 months of CI treatment, the M-HAQ ADL difficulty scale had improved 27%, from a baseline value of 0.66 ± 0.14 (mean \pm SEM) to a value of 0.48 ± 0.14 ($P < 0.05$). The MRSS declined steadily, and after 12 months of CI treatment, had decreased by 23%, from a baseline value of 26.35 ± 2.35 to a value of 20.29 ± 2.53 ($P < 0.005$) (Figure 3B). In the patients with diffuse SSc, the MRSS decreased by 26.6% after 12 months of CI treatment, from a baseline value of 28.6 ± 2.5 to a value of 21.0 ± 2.7 ($P < 0.005$) (results not shown). There were no significant correlations between MRSS score or M-HAQ score and decreases in IL-10 or IFN γ production by PBMC cultured with CI α chains after 12 months of CI treatment (results not shown).

Because of patient noncompliance and scheduling problems, only 11 of the 17 patients who finished the study had DLco measurements and PFTs performed at 0 and 12 months. For these 11 patients, the mean DLco, corrected for alveolar volume and hemoglobin, increased by 9.58% from 3.34 to 3.66 ml/minute/mm Hg (*P*



A



B

Figure 3. A, The modified Rodnan skin thickness score (MRSS) and B, the modified Health Assessment Questionnaire (M-HAQ) scores in patients with systemic sclerosis. The MRSS and the M-HAQ were measured before and after 12 months of oral treatment with bovine type I collagen (CI). Values are the mean and SEM. *P* values determined by Student's paired *t*-test; NS = not significant. ADL = activities of daily living.

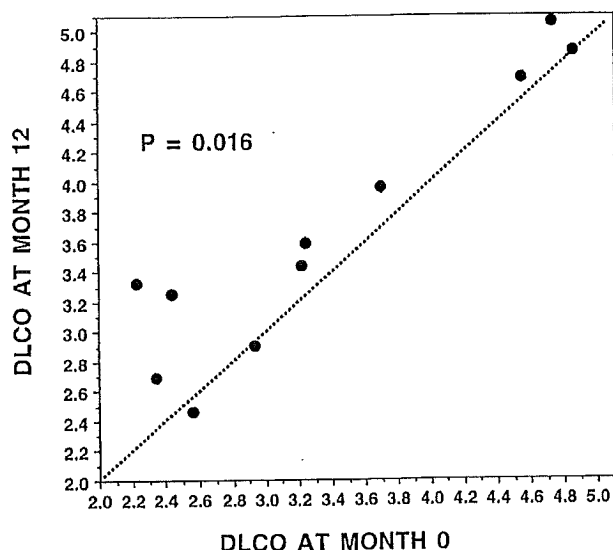


Figure 4. Diffusing capacity for carbon monoxide (DLCO) in 11 patients with systemic sclerosis, before and after 12 months of oral treatment with bovine type I collagen. The dotted line is a reference line with a slope of 1. *P* value determined by Student's paired *t*-test.

< 0.016) after 12 months of CI therapy (Figure 4). There was no significant change in forced vital capacity or in serum creatinine values.

DISCUSSION

In patients with limited and diffuse SSc, the administration of oral bovine CI for 1 month at a dosage of 100 μ g/day and for 11 months at 500 μ g/day resulted in significant reductions in IFN γ and IL-10 production by CI α -chain-stimulated PBMC. Levels of serum sIL-2R were also significantly reduced after induction of oral tolerance to CI. This reduction in sIL-2R, although small, suggests that overall T cell stimulation was reduced. To our knowledge, levels of sIL-2R in sera have not been reported in other studies of human oral tolerance or in animal models of oral tolerance. Therefore, the significance of this modest reduction in serum sIL-2R levels in the context of oral tolerance is not known at present. Taken together, these data indicate that oral tolerance to CI was effected by this treatment regimen.

The mechanism(s) by which this oral CI regimen induced these immune changes is not readily apparent. It is likely that IFN γ production by CI α -chain-stimulated PBMC is largely from CD4 $^{+}$ Th1 cells; but natural killer cells are also a potential source of this

cytokine. The reduced production of IFN γ by PBMC CD4 $^{+}$ T cells could be due to 1 or a combination of the 3 mechanisms of oral tolerance induction (i.e., suppressive regulatory T cells, clonal anergy, or clonal deletion) (10–12).

The reduced IL-10 production by SSc CI α -chain-stimulated PBMC after oral CI treatment was unexpected, given that IL-10 has been reported to be up-regulated in peripheral lymphoid tissue or in target organs in autoimmune immune models after oral tolerance induction by low-dose antigen (20). However, in some circumstances, IL-10 can be produced by Th1 cells, and in humans, there is less rigidity to the Th1/Th2 paradigm originally described using clonal mouse T cells (20–22). In addition, IL-10 is produced by cells other than CD4 $^{+}$ T cells (23,24). Monocyte/macrophages are a major source of this cytokine (23,25). The reduced IL-10 production by CI α -chain-stimulated SSc PBMC after oral CI treatment may reflect overall decreased T cell responsiveness to the α chains, and therefore decreased stimulation to monocytes by IFN γ or other cytokines from T cells that up-regulate IL-10 production by monocytes. Finally, published studies of oral tolerance in animal models have not measured antigen-stimulated PBMC production of IL-10, or other cytokines elaborated by PBMC, before and after oral tolerance induction. The published studies of animal models of oral tolerance all measure cytokine expression in lymphoid tissue or target organs rather than peripheral blood.

NSAIDs are known to inhibit oral tolerance in animal models and may be a confounding factor in human oral tolerance (26–28). For this reason, we advised the 5 patients who had been taking NSAIDs for the first 6 months of the trial to discontinue them for the last 6 months of the trial, which they did.

Although there were significant improvements in the MRSS and M-HAQ scores, these findings should be viewed with caution, since this was an open-label study and these changes may reflect variations or spontaneous changes in the disease or a placebo effect. The DLco values, while showing statistically significant improvement, are still just below the clinically significant cutoff of $\geq 10\%$. Clearly, a larger population of more homogeneous patients with diffuse SSc needs to be evaluated in a randomized, double-blind, placebo-controlled study before it can be categorically concluded that oral CI tolerance induction ameliorates the SSc disease process.

One could speculate that the mechanism by which oral CI might possibly ameliorate SSc could involve anergy and/or suppression depending on the

dose of CI given. Although the 500 $\mu\text{g/day}$ dosage of CI induced oral tolerance, clearly other dosages need to be studied. The feeding of CI to SSc patients could anergize autoreactive cells and/or generate major histocompatibility complex class I- or class II-restricted regulatory T cells that sequester in involved tissues, where they release small amounts of immunosuppressive cytokines (IL-4, IL-10, TGF β 1) that down-regulate autoaggressive cells by the mechanism of antigen-driven bystander suppression. By antigen-driven bystander suppression, these CI-specific T cells could down-regulate T cell interactions with other antigens (29), as has been demonstrated in autoimmune animal models in which oral tolerance has been induced by oral administration of antigens from organs that are the target of attack.

These animal models provide a theoretical basis for predicting that in SSc patients, CI, although it may not be an initiating antigen of SSc or even be involved in its pathogenesis, when given as an oral tolerogen, may well suppress T cell-mediated fibrogenesis by suppressing activated T cells. If activated CD4+ T cells present in the tissues of SSc patients could be down-regulated, then with time, the fibrogenic phenotype of SSc fibroblasts might revert to normal. It is known that after serial passage of SSc fibroblasts in vitro for several generations, they regain a more normal phenotype with regard to matrix synthesis (30). Also, patients with longstanding SSc tend to have less skin thickening and collagen deposition than they had in earlier stages of their disease.

While IL-4 and TGF β at high concentrations (~50 ng/ml and 5 ng/ml, respectively) can up-regulate collagen synthesis by cultured fibroblasts in vitro, lower concentrations (e.g., TGF β 1 at 1,000 times less) are capable of modulating immune cells (30-34). The fact that oral CI treatment did not increase skin or lung fibrosis suggests that if GALT-derived regulatory T cells producing these cytokines were generated by oral CI treatment, the levels of these cytokines are likely to be lower than is required to trigger collagen synthesis by fibroblasts.

Oral CI administration appears to be safe in SSc patients. Its efficacy needs to be assessed by a larger placebo-controlled, double-blind trial.

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Errata

In the article by García-Porrúa et al published in the March 2000 issue of *Arthritis & Rheumatism* (pp 584-592), there was an error in the second full sentence in the left column on page 589. The sentence should have read, "Six of 39 patients diagnosed as having idiopathic EN (15.4%) had 1 or more predictive factors for secondary EN, and 64 of 67 patients diagnosed as having secondary EN (95.5%) had 1 or more predictive factors for secondary EN" [emphasis added]. This is also how the first sentence of the first footnote in Table 4 should have read.

In the article by van der Heijden et al in the March 2000 issue (pp 593-598), the reference cited at the end of the first sentence in the second paragraph of Patients and Methods (page 594) should have been reference 11, rather than reference 4. The reference cited at the end of line 12 in the second paragraph of the Discussion (page 597) should also have been reference 11, rather than reference 8.

We regret the errors.

EXHIBIT 5

Transgenic plants expressing autoantigens fed to mice to induce oral immune tolerance

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Oral administration of protein can induce antigen-specific immune hyporesponsiveness¹. However, the utility of oral tolerance to autoantigens in the treatment of autoimmune diseases may be limited when candidate autoantigens cannot be produced by conventional systems in quantities sufficient for clinical studies. Plants may be ideally suited for this purpose, as they can synthesize, glycosylate and assemble mammalian proteins to provide huge quantities of relatively low cost soluble proteins². Furthermore, edible transgenic plants could provide a simple and direct method of autoantigen delivery for oral tolerance. Therefore, the aim of this study was to determine whether a transgenic plant expression system was capable of synthesizing the diabetes-associated autoantigen, glutamic acid decarboxylase (GAD)^{3,4} in an immunogenic form and whether the oral administration of an autoantigen expressed by a plant could directly induce protective immune responses in a mouse model of diabetes. We show that a GAD-expressing transgenic plant, given as a dietary supplement, inhibits the development of diabetes in the non-obese diabetic (NOD) mouse.

Glutamic acid decarboxylase (GAD) has been implicated as an autoantigen in diabetes, as anti-GAD antibodies appear before the onset of disease both in patients and in non-obese diabetic (NOD) mice, a mouse model of spontaneous insulin-dependent diabetes mellitus^{5,6}. Minimally invasive and antigen-specific therapies, such as oral tolerance to GAD for the prevention of diabetes, would offer considerable advantages over current therapy for the disease. Several reports have shown that immunization of young NOD mice with GAD suppresses diabetes^{7,8,9}, but as oral tolerance requires considerably larger amounts of protein, the role of oral GAD in the prevention of diabetes has not been tested. Poor GAD protein solubility in bacteria and inadequate production capacity from mammalian cells have precluded the use of these conventional systems for large-scale production of GAD for long-term oral tolerance studies^{7,10,11}.

To address these problems, we created transgenic plants expressing full-length GAD67. Mammalian GAD is represented by two isoforms, with GAD65 representing the major form of GAD in rat and human pancreatic islets¹² and GAD67 predominating in mouse islets. To permit feeding of transgenic plant tissue directly, GAD67 cDNA was transferred into low-alkaloid tobacco (*Nicotiana tabacum* cv. *Lonibow*) and potato plants (*Solanum tuberosum* cv. *Desiree*), which were well tolerated by mice. The expression vector used an enhanced CaMV 35S promoter with a 5' untranslated

leader sequence derived from tobacco etch potyvirus to maximize expression (Fig. 1a) and was transferred into plants by *Agrobacterium tumefaciens*-mediated transformation. Insertion was confirmed by Southern blot, and northern blots showed GAD67 mRNA transcripts with the expected size of 2 kilobases (not shown). There was variation in steady-state levels of mouse GAD67 mRNA transcripts among the individual transgenic lines; this variation is consistent with the random nuclear insertion of transferred DNA with *Agrobacterium*-mediated transformation. No endogenous GAD mRNA homologues were detected in untransformed control plants or control plants into which expression vector alone was inserted. Western blots of unpurified protein extracts from transgenic plant tissue showed a single protein band of the correct size (Fig. 1b), and plants expressing high levels of GAD67 protein were maintained as stable plant lines. The level of GAD expression was estimated from blot densitometry and found to be approximately 0.4% of total soluble proteins. GAD67 expression was similar in tobacco leaves and potato tubers and was not detected in untransformed control plants or control plants into which expression vector was inserted.

Mechanisms of oral tolerance include deletion of antigen-reactive T cells, induction of anergy, alteration of Th1/Th2-type helper T-cell responses and active suppression^{13,14}. We tested the capacity of plant tissue expressing GAD67 given as a dietary supplement to alter T-cell responses in NOD mice. Supplementation was calculated to deliver approximately 1–1.5 mg of GAD daily, a dosage based on oral tolerance studies of NOD mice with insulin¹⁵. After 4 weeks of diet supplementation, GAD67 induced proliferation of spleen cells from control plant-fed mice *in vitro*, whereas proliferation of spleen cells from mice fed plant GAD67 for 4 weeks was reduced to that of controls with medium only (Fig. 2a). No proliferative responses occurred with an irrelevant protein (OVA) in cultures. We also assessed anti-GAD antibody responses in treated mice. Although serum levels of total anti-GAD IgG antibodies in control mice did not change or increased slightly in GAD67-fed mice, challenge with purified GAD67 in the foot pad resulted in a twofold increase in anti-GAD IgG antibody in GAD67-fed mice compared with control mice [absorbance units (A_{405}) = 0.40 ± 0.05 vs. 0.21 ± 0.05 , respectively; $n = 3$ per group].

Oral tolerance has been shown to bias T cells toward the development of Th2-type responses when they are reexposed to antigen¹, which can result in predominantly IgG1 rather than IgG2a antibody responses¹⁶. Both GAD67 and control mice showed an

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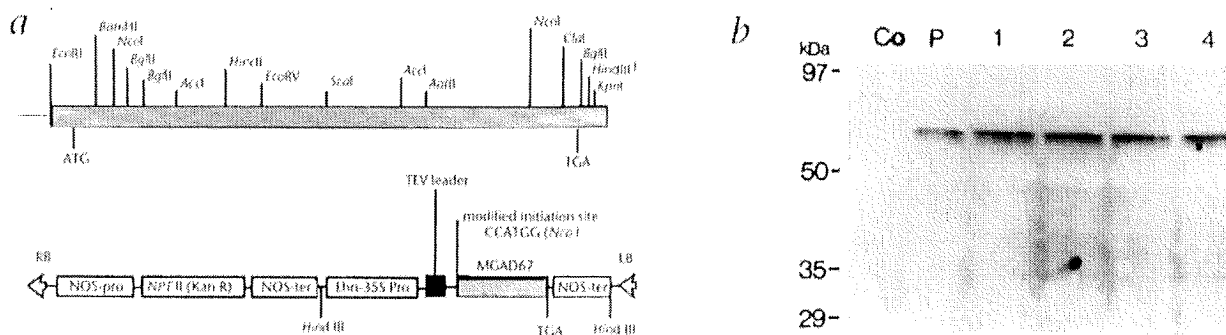


Fig. 1 *a*, Schematic representation of the plant transformation and expression vector. Restriction sites of mouse GAD67 have been revised from the original published sequence¹¹. The coding sequence is indicated as ATG (start) and TGA (stop). The GAD67 sequence was inserted between the CaMV 35S promoter and the transcription-termination signal of nopaline synthase (NOS). A new NcoI restriction site was created to form part of the translational start site. Abbreviations: Enh-35S, enhanced 35S promoter, which includes a duplicated enhancer; TEV leader, leader sequence of tobacco etch virus; MGAD67, the DNA sequence for mouse glutamate decarboxylase; NOS-ter, the DNA sequence of the nopaline synthase 3' terminator; *nptII*, neomycin phosphotransferase II gene; RB, right border; LB, left border. *b*, Western blot of murine GAD67 expression in transgenic tobacco plants. Protein (30 µg) extracted from leaves was separated on a 12% SDS-polyacrylamide gel, and GAD67 was identified by anti-GAD antibody. Lanes are as marked: Co, extracts from a control plant; P, purified mouse GAD67 (100 ng); lanes 1–4: extracts from independent transgenic plants. Protein size standards are indicated on the left side of the gel.

increase in serum IgG1 anti-GAD antibodies after challenge with GAD67 (Fig. 2*b*), but GAD67-fed mice consistently developed 2- to 12-fold increases in anti-GAD IgG1 antibody over controls, with levels equivalent to those in NOD mice receiving intraperitoneal priming of purified GAD67 (ref. 7 and unpublished data). No changes in IgG2a anti-GAD antibodies were found in any group. We also tested supernatants from splenocyte cultures for the expression of cytokines associated with Th1 [interferon-γ (IFN-γ)] or Th2 (the interleukins IL-4, IL-10) in response to soluble, purified GAD67. Consistent with previous reports in NOD mice after systemic or nasal administration of GAD protein, IFN-γ levels were reduced in GAD67-fed mice [$A_{405} = 2.1 \pm 0.2$ vs. 1.4 ± 0.1 ; $n = 7$ per group; $P = 0.02$] with concurrent increases in both IL-4 and IL-10 levels ($P = 0.1$)^{3,7-9,16}. Although these data suggest that immune responses in GAD67-fed mice may be biased toward the development of GAD67-specific Th2 responses, they do not exclude other mechanisms associated with oral tolerance, including apoptotic deletion of antigen reactive cells, induction of anergy, and active suppression^{13,14}.

We then tested whether a simplified approach to oral tolerance using transgenic plant tissue expressing GAD67 and direct dietary supplementation could prevent diabetes in NOD mice. Vector-transformed plants were used for controls to exclude any potential influence of transformation alone on these results. Mice were supplemented from 5 weeks to 8 months of age ($n = 12$ per group). Ten out of 12 NOD mice fed either tobacco ($n = 6$) or potato GAD67 ($n = 6$) remained free of disease ($P = 0.007$ from controls) with equivalent protection using tobacco or potato. In contrast, 8 out of 12 control plant-fed mice, equally divided between tobacco and potato supplementation, developed diabetes (Fig. 3). The mice tolerated plant tissue well, and no differences between groups were observed in the appearance or weight gain of mice. Although it is difficult to compare results between different models, these results — which are comparable to those previously reported with other methods of GAD immunization — suggest that oral plant GAD67 may offer an effective alternative approach to treatment^{3,7,9,15}.

In summary, we present the first report of a diabetes-related autoantigen (GAD67) to be expressed in transgenic plants,

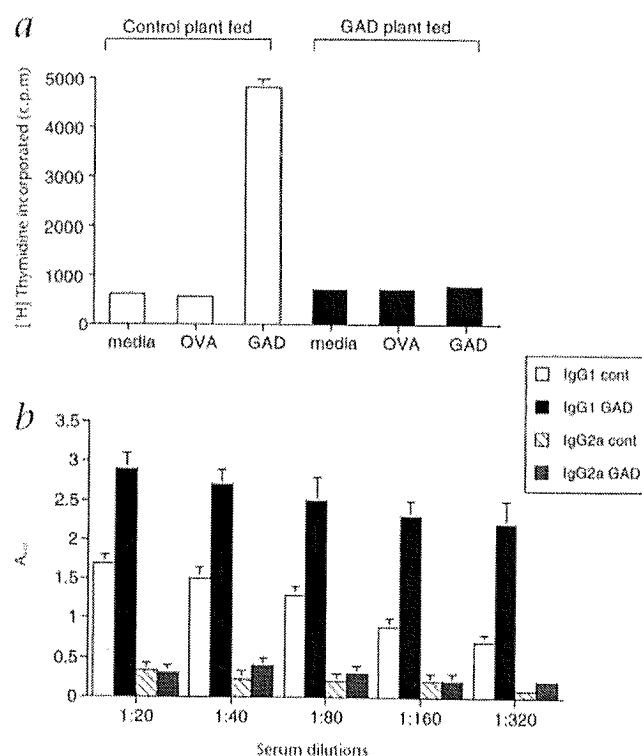


Fig. 2 *a*, Loss of GAD-specific T-cell proliferation in plant GAD-fed mice. Female NOD mice were fed either plant GAD67 or control plant and challenged in the foot pad with 50 µg purified GAD67 after 4 weeks of feeding. Proliferative responses of splenocytes to GAD67 and OVA were determined after 10 days, and results shown are representative of three experiments performed using two mice per group. GAD-fed mice had responses to GAD67 equivalent to medium alone, and a marked difference was seen between GAD- and control plant-fed ($SI = 7.6$) mice ($P < 0.001$). *b*, Mice fed GAD67-producing or control plants were assessed for serum anti-GAD IgG antibody isotypes by ELISA. Values (means \pm s.e.m.) of grouped samples ($n = 4$) are presented as means of A_{405} at various dilutions. IgG1 anti-GAD antibody levels were different between GAD67- and control plant-fed mice at all dilutions ($P < 0.002$). Results are representative of three experiments.

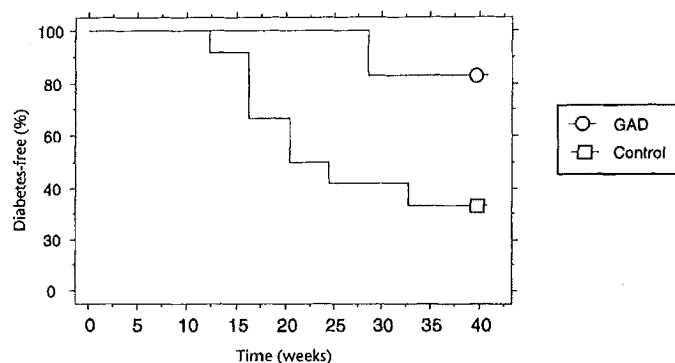


Fig. 3 Actuarial analysis of survival of mice versus the development of diabetes. Female NOD mice ($n = 12$ per group) were fed plant GAD67 or control plant from the age of 5 weeks as described in the Methods. Mice were followed for the onset of diabetes. Incidence of diabetes in GAD67- and control plant-fed mice differed by log rank analysis ($P = 0.007$).

which, given as a dietary supplement, inhibits the development of diabetes in NOD mice. Expression of mouse GAD67 in plants is stable, although levels may be made optimal with targeting to specific cellular compartments or cell types¹⁷. Concerns have been raised recently regarding the potential acceleration of autoimmune disease by oral administration of autoantigen proteins^{18,19}. Although the issue of potential acceleration of disease needs to be carefully considered in any clinical trials, a similar approach using human GAD65 expressed in edible transgenic plants may be useful in individuals at risk for developing diabetes. Although the availability of GAD protein from transgenic plants in large quantities will facilitate further studies of its role in the pathogenesis of diabetes, plants may also be useful in the production of many other candidate mammalian proteins to induce oral tolerance in autoimmune diseases and transplantation.

Methods

Construction of plants expressing mouse GAD67. A revised restriction map based on the mouse GAD67 cDNA clone²¹ is shown in Fig. 1a. The coding sequence of GAD67 cDNA was modified to introduce an *NcoI* site as part of the ATG start codon. The modified GAD67 was used to replace the β -glucuronidase (GUS) coding region in pTRL2-GUS composed of a CaMV 35S promoter with a double enhancer sequence (Ehn-35S) linked to a 5' untranslated tobacco etch virus leader sequence, GUS and a nopaline synthase (NOS) terminator²⁰. The resulting expression cassette was inserted into the binary vector pBIN19, and the resulting pSM215 was transferred into *Agrobacterium tumefaciens*²¹. Transformation of low-alkaloid tobacco (cv. *Lonibow*) and potato (*Solanum tuberosum* cv. *Desiree*) was by leaf-disc cocultivation with *A. tumefaciens* LBA4404 containing GAD plasmid pSM215 (Fig. 1a) or pBIN19 alone, using a method described for tobacco²² and potato²³. Mature plants were regenerated from shoots under antibiotic selection in hormone-free rooting medium, and high-expression plants were selected for long-term maintenance.

Western blot and tissue printing. Soluble proteins from transgenic plants were extracted in buffer with protease inhibitors and quantified with bovine serum albumin (BSA). Samples were applied to 12% acrylamide SDS-PAGE gels²⁴. Detection was by an enhanced chemiluminescence method (Boehringer Mannheim) after incubation with anti-GAD antibody⁷ and a horseradish peroxidase-conjugated secondary antibody. For scanning densitometry of blots we used Image 1.41 (National Institutes of Health, Bethesda, MD) on a Macintosh PowerPC computer (Cupertino, CA).

Oral feeding trials. NOD mice were housed according to guidelines of the Canadian Council on Animal Care and were screened for bacterial and viral pathogens. Parental colony incidence of diabetes in female mice is 70–80%

by 30 weeks. Female NOD mice were started at 5 weeks of age. Fresh GAD67 potato tuber slices were added to feed (approximately 3 g per mouse) or freeze-dried GAD67 tobacco leaves were mixed into feed (1 g per mouse) offered to cages of two to four mice, and the amount was calculated to deliver approximately 1–1.5 mg of GAD67 per mouse daily if completely ingested. Control mice received an equivalent amount of corresponding plant tissue from vector-transformed plants, and on most days the supplement was completely consumed. Glucose levels were monitored weekly in urine by using TES-TAPE (Lilly, Indianapolis, IN), with serum testing to confirm diabetes (>16.7 mmol/l on two consecutive days; Glucoscan 2000 Strips, Lifescan, Milpitas, CA).

T-cell proliferation and cytokine assays. NOD mice were challenged with 50 μ g of highly purified *Escherichia coli*-derived recombinant GAD67 (MGAD)⁷, combined with incomplete Freund's adjuvant (IFA; Sigma Chemical Co.), injected into each hind foot pad, after 4 weeks of supplementation. Ten days later mice were killed, and a single-cell suspension of lymph node or spleen cells was cultured in 96-well flat-bottomed plates (Becton Dickinson, NJ) at 2×10^5 cells/well in RPMI (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (Gibco, Grand Island, NY). Cultures were stimulated with purified MGAD (10–20 μ g/ml) or OVA (10 μ g/ml, Sigma) as a negative control. Cultures were incubated for 72 h before pulsing with [³H]thymidine (1 μ Ci/well; Du Pont-NEN, Boston, MA) and counting (LKB Instruments, Gaithersburg, MD). Proliferation was considered positive if the stimulation index (SI) was greater than 3.0 and the change in counts per minute from control (medium alone) was >500 . For cytokine assays, spleen cells (4×10^5 /well) were cultured for 24 or 48 h in 24-well flat-bottomed plates with 2 ml of RPMI with FCS, anti-CD3 mAb (145.2C11, 5 μ g/ml) and purified mouse GAD67 (10 μ g/ml). Supernatants were tested for IFN- γ , IL-4 and IL-10 by standard sandwich ELISA using a kit (PharMingen, San Diego, CA), and results are expressed as arbitrary absorbance units at 405 nm (A_{405}) for group comparisons.

Anti-GAD antibody isotyping. Mice used for T-cell proliferation assays were assessed for anti-GAD antibody levels by ELISA as previously described⁷ with anti-isotype (IgG1, IgG2a) antibody (Becton Dickinson, Mississauga, Ont.). Data are presented as means of A_{405} of grouped samples.

Statistics. Data are expressed as means \pm s.e.m. Statistical analyses of grouped data were carried out using analysis of variance (ANOVA). Differences in the incidence of diabetes between treatment groups were analyzed by actuarial survival to the time of diabetes and log rank tests (Statview, Abacus, Berkeley, CA).

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EXHIBIT 6

AN 1999:597309 PROMT
TI AutoImmune shares collapse on Colloral data in rheumatoid arthritis.
SO Marketletter, (13 Sep 1999) .
ISSN: 0951-3175.

PB Marketletter Publications Ltd.

DT Newsletter

LA English

WC 756

TX US biotechnology firm AutoImmune saw its shares crash 74% on September 1 to close at \$1.40 following the announcement that its oral tolerance drug Colloral (collagen) had failed in Phase III development. By the end of the trading week (September 3), the firm's stock was selling at a miserly \$0.84, a nasty turnaround for a company which was riding high on the back of its oral tolerance technology a couple of years ago with stock being traded around the \$14 mark.

AutoImmune says that Colloral will be dropped from development and the firm will "immediately reduce its headcount and other operating expenses to conserve resources as we evaluate our strategic options to maximize shareholder value." The company told the Marketletter that it plans to cut its workforce by 96%, downsizing to eight staff from 26 immediately and then to two employees by the end of the month.

In the 772-patient trial, Colloral was found to be safe but did not meet the primary endpoint, which the spokeswoman said was achieving statistical significance in three out of the "core-four" parameters (tender joints, swollen joints, physician's global assessment and patient global assessment). While AutoImmune says that "substantial improvements" from baseline were observed in each of these measurements, the placebo response was "much greater than previously observed." In fact, the spokeswoman noted that, although the data were not publicly available at present, the placebo response was two times higher than in previous studies of the drug. Full data may be presented at a forthcoming rheumatology meeting, and the firm is considering switching the focus of Colloral to a nutraceutical product.

When asked whether the trial could be designed differently, the spokeswoman told the Marketletter that it "was perfect." Financially, however, the firm cannot keep funding the clinical development of Colloral. AutoImmune had continued its clinical development of the drug even though earlier trials had failed to demonstrate strong data. Two years ago, the company revealed that two Phase II trials of Colloral in RA had failed to yield statistically significant results (Marketletter May 19, 1997). However, the firm decided to pursue Phase III development following an independent re-analysis by statisticians who concluded that the drug was significantly more effective than placebo (Marketletter September 15, 1997).

General expectations for the drug were not high, particularly following the earlier failure of another mucosal tolerance program, **Myloral** (myelin basic protein) for multiple sclerosis which performed no better than placebo in Phase III trials.

Yet some investors may see this a good buying opportunity, with analysts pointing out that the company has a decent cash position with few liabilities; as of June 30, the firm had cash and cash equivalents of almost \$9.7 million and the spokeswoman added that once liabilities have been paid, this will be down to around \$7 million.

Ideal opportunity to buy?

Furthermore, AutoImmune has a very strong intellectual property position and is still conducting a number of other trials which are funded

externally. These include studies in new-onset type 1 diabetes (with Eli Lilly) and a pilot trial in chronic organ transplant rejection (results from both are due next year). Enrollment is continuing in a National Institutes of Health-funded long-term prevention study for type 1 diabetes.

Importantly, the firm also has an exclusive agreement with Teva Pharmaceutical for applications of AutoImmune's proprietary technology. The deal covers the development of an oral formulation of Teva's injectable multiple sclerosis drug Copaxone (glatiramer acetate) and an oral product for the treatment of myasthenia gravis, for which AutoImmune will receive milestone payments on product approval and royalties on any future sales. Teva is getting ready to start a Phase II/III trial of oral Copaxone with the first patient expected to be enrolled by year end, while the product for myasthenia gravis is also due to begin clinical development before the end of the year.

Despite speculation that the company's faith in the potential of inducing oral tolerance to antigens as a means of treating autoimmune disease may be misguided, AutoImmune says it still firmly believes in its technology. In a statement, the company said that "both basic and clinical research focused on enhancing the biological effect of [mucosal tolerance therapy] in patients will continue."

AutoImmune is currently assessing a number of different plans, including possible mergers and converting into a shell company while waiting for clinical data from its other ongoing trials to come through. The spokeswoman said that there has been interest from some firms in a merger, particularly as AutoImmune has such a strong IP position.

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CC *EC750 Securities prices
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ICL *INTL Business, International
NAIC *325412 Pharmaceutical Preparation Manufacturing
GT *CC1USA United States
FEAT LOB; COMPANY

EXHIBIT 7

Rapid communication

No effect of oral insulin on residual beta-cell function in recent-onset Type I diabetes (the IMDIAB VII)

P. Pozzilli, D. Pitorro, N. Visalli, M. G. Cavallo, R. Buzzetti, A. Crinò, S. Spera, C. Suraci, G. Multari, M. Cervoni, M. L. Manca Bitti, M. C. Matteoli, G. Marietti, F. Ferrazzoli, M. R. Cassone Faldetta, C. Giordano, M. Shriglia, E. Sarugerl, G. Ghirlanda and the IMDIAB Group*

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Abstract

Aims/hypothesis. Induction of tolerance to insulin is achievable in animal models of Type I (insulin-dependent) Diabetes mellitus by oral treatment with this hormone, which can lead to prevention of the disease. In the Diabetes Prevention Trial of Type I diabetes (DPT-1), oral insulin is given with the aim of preventing disease 'insurgence'. We investigated whether if given at diagnosis of Type I diabetes in humans, oral insulin can still act as a tolerogen and therefore preserve residual beta-cell function, which is known to be substantial at diagnosis.

Methods. A double-blind trial was carried out in patients (mean age \pm SD: 14 ± 8 years) with recent-onset Type I diabetes to whom oral insulin (5 mg daily) or placebo was given for 12 months in addition to intensive subcutaneous insulin therapy. A total of 82 patients with clinical Type I diabetes (< 4 weeks duration) were studied. Basal C peptide and glycated haemoglobin were measured and the insulin requirement monitored every 3 months up to 1 year. Insulin antibodies were also measured in 27 patients treated with oral insulin and in 18 patients receiving placebo

at the beginning of the trial and after 3, 6 and 12 months of treatment.

Results. The trial was completed by 80 patients. Overall and without distinction between age at diagnosis, at 3, 6, 9 and 12 months baseline mean C-peptide secretion in patients treated with oral insulin did not differ from that of those patients treated with placebo. In patients younger than 15 years a tendency for lower C-peptide values at 9 and 12 months was observed in the oral insulin group. Insulin requirement at 1 year was similar between the two groups as well as the percentage of glycated haemoglobin. Finally, IgG insulin antibodies were similar in the two groups at each time point.

Conclusion/interpretation. The results of this study indicate that the addition of 5 mg of oral insulin does not modify the course of the disease in the first year after diagnosis and probably does not statistically affect the humoral immune response against insulin. [Diabetologia (2000) 43: 1000–1004]

Keywords Type I diabetes, oral insulin, insulin antibodies, prevention.

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Abbreviations: DPT-1, Diabetes Prevention Trial of Type I diabetes; IA, insulin antibodies

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The use of insulin, given either subcutaneously or orally, in subjects at risk for Type I diabetes has been recently introduced in the Diabetes Prevention Trial of Type I diabetes (DPT-1) trial, a large multinational trial in the United States, with the aim of preventing the destruction of beta cells and the clinical onset of the disease [1]. The rationale for the use of insulin in these patients is to induce beta-cell rest and/or tolerance to the hormone and its peptides [2] which are thought to be important targets of the autoimmune response leading to beta-cell destruction [3]. In ani-

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Table 1. Baseline clinical characteristics and metabolic control at entry of the trial in the two groups of patients

	Oral insulin	Placebo
Number of patients	46	36
Sex (number of males)	25	17
Age (years \pm SD)	14.1 \pm 7.9	13.8 \pm 7.6
Duration of symptoms before diagnosis (days)	34.5 \pm 34.6	38.8 \pm 42.5
Blood glucose at diagnosis (mmol/l)	20.7 \pm 9.4	22.1 \pm 8.3
Insulin dose (U \cdot kg ⁻¹ \cdot day ⁻¹) \pm SD	0.63 \pm 0.3	0.55 \pm 0.3
Glycated haemoglobin (%) \pm SD	9.9 \pm 0.5	9.0 \pm 0.5
Basal C peptide (pmol/l) \pm SD	0.23 \pm 0.3	0.17 \pm 0.15

Values between groups are not statistically different

mal models, this approach was shown to be effective in halting the process leading to Type I (insulin-dependent) diabetes mellitus [4]. Furthermore, oral insulin was able to reduce the extent and modify the type of lymphocytic infiltration in the pancreas of susceptible mice [5].

The International Diabetes Immunotherapy Group suggested that approaches to prevent Type I diabetes should first be tested in recent-onset Type I diabetic patients and, if effective, applied to pre-diabetic people [6]. In our study we evaluated the effects of oral insulin treatment at clinical onset of Type I diabetes, which could aid in reducing the further destruction of beta cells that generally occurs within the first 12 months after diagnosis. In patients with recent-onset Type I diabetes, simultaneous treatment with subcutaneous and oral insulin might have considerable effects, as the former could improve metabolic control and the latter induce tolerance. As disturbances in the gut immune reactivity could be relevant in the pathogenesis of Type I diabetes [7], induction of oral tolerance with a specific antigen, such as insulin, could be appropriate for this disease. It is therefore the aim of this double-blind randomized trial to find out whether treating patients who have recent-onset Type I diabetes with oral insulin in addition to identified subcutaneous insulin therapy [8] could improve metabolic control, as measured by glycated haemoglobin value, insulin dose and C peptide concentration. The effects of such treatment on the rate of spontaneous clinical remission (suspension of insulin therapy) and on the extent of humoral immune response against insulin were also evaluated.

Subjects and methods

Selection of patients. Patients with recent-onset Type I diabetes ($n = 82$) were recruited by 8 participating centres of the IMDIAB Group and 1 affiliated centre. Each centre contributed with nearly equal numbers of patients to the study. Inclusion criteria were the following: (1) diagnosis of the disease according to the World Health Organisation (WHO) criteria, with age at presentation between 5 and 35 years, (2) duration of clinical disease (since the beginning of insulin therapy) less than 4 weeks, (3) no medical contra-indications (including

pregnancy) or any other major chronic disease, (4) willingness and capability to participate in regular follow-up.

Patients' baseline clinical characteristics and metabolic control at entry of the trial are shown in Table 1.

Study design and treatment protocol. The study was endorsed by the Italian Ministry of Health and approved by the central ethics committee at the Gemelli Policlinic, The Catholic University of the Sacred Heart, Rome. After informed consent had been obtained and baseline measurements completed, a permuted-block design was used to blindly assign patients to each of the two treatment groups. A random number table was adopted with a prepared list and a randomization code was assigned to each participating centre. Of the patients 46 received 5 mg daily of oral insulin and 36 placebo. Oral treatment began within 4 weeks of diagnosis in both groups and lasted 12 months. All patients also received intensive subcutaneous insulin therapy as soon as possible after diagnosis to optimize metabolic control and maintain blood glucose concentrations as near to normal as possible (see below).

Guidelines for insulin therapy. All participating centres used the same treatment protocol as in our previous IMDIAB trials [9, 10] based on the following rules: if pre-prandial blood glucose values were below 6.5 mmol/l, the insulin dose was decreased by 10%; if blood glucose concentrations were consistently below 4.5 mmol/l for more than 3 days the insulin dose was decreased by 20%. Insulin therapy was not discontinued unless 2-h postprandial blood glucose concentrations measured at home were consistently below 7.5 mmol/l. Patients with blood glucose above 10 mmol/l received a 10% increase in insulin dose or had their insulin regimen modified. Frequent telephone consultations were arranged with patients to adjust the insulin dose as required.

Investigations and follow-up. Patients included in the study were followed up by the staff of the centre where they were enrolled. Patients were started on a 55% carbohydrate diet and received three to four injections daily of regular plus intermediate insulin. Each patient recorded capillary glucose concentration at fasting and before and after meals daily, for a total of at least 20 weeks. The subcutaneous insulin dose was adjusted to obtain near-normal blood glucose concentrations.

Patients were examined weekly for the first month of therapy and then monthly by the same team of physicians in each participating centre. Drug toxicity was evaluated at follow-up visits, by liver and renal function tests and total blood count. Glycated haemoglobin (HbA_{1c}) (normal range 4-7%) was measured every 3 months by a column assay (Bio-Rad, Milan, Italy), and basal C peptide concentration was evaluated after euglycaemia was achieved before entry into the trial, and at 3-monthly intervals for 1 year thereafter. C-peptide concentra-

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Table 2. Metabolic outcomes during follow-up

	Oral insulin	Placebo
Number of patients	44	36
Insulin dose (U/kg) \pm SD		
3 months	0.44 \pm 0.3	0.37 \pm 0.2
6 months	0.48 \pm 0.3	0.43 \pm 0.2
9 months	0.54 \pm 0.3	0.52 \pm 0.3
12 months	0.61 \pm 0.2	0.58 \pm 0.3
Glycated haemoglobin (%) \pm SD		
3 months	6.2 \pm 1.8	5.8 \pm 1.5
6 months	6.5 \pm 1.5	6.3 \pm 1.5
9 months	7.1 \pm 1.6	7.1 \pm 1.5
12 months	7.6 \pm 1.3	7.1 \pm 1.5
Basal C-peptide (nmol/l) \pm SD		
3 months	0.30 \pm 0.2	0.30 \pm 0.2
6 months	0.30 \pm 0.2	0.30 \pm 0.2
9 months	0.20 \pm 0.2	0.25 \pm 0.2
12 months	0.17 \pm 0.2	0.22 \pm 0.2

Values between the two groups are not statistically different for insulin dose, HbA_{1c}, C-peptide concentration

tion was measured by radioimmunoassay, using a commercially available kit (Bio-Rad). The normal range of fasting C-peptide established in 150 control subjects, 71 females and 79 males, aged 5–40 years, median 18 years, with no family history of Type 1 diabetes was 0.35–1 nmol/l with intracoefficients and intercoefficients varying between 10% and 15%, respectively.

Insulin antibodies. Insulin antibodies (IA), expressed as a concentration of units/5 μ l serum, were measured in serum samples drawn from 27 patients treated with oral insulin and 18 receiving placebo at the beginning of the trial and after 3, 6 and 12 months of treatment and stored at -20°C . A modification of the micro-radio-binding assay of Williams [11] was used as described previously [12]. The threshold and 99th centile of 97 control subjects, 51 females and 46 males, aged 2–48 years, median 21 years, with no family history of Type 1 diabetes, was calculated at greater than 4.4 insulin antibody units.

Evaluation of response to therapy. Response to therapy was monitored throughout the study by investigating the occurrence of clinical (complete) remission defined, according to the recommendations of the International Diabetes Immunotherapy Group (IDIG), as restoration of normal fasting and postprandial blood glucose concentration without any insulin treatment for more than 2 weeks [6]. Moreover, metabolic control (C-peptide, HbA_{1c} and insulin dose) was evaluated at 3-monthly intervals.

Sample size and statistical analysis. The number of patients to be included in the study was calculated from an analysis of results of trials published in the past (courtesy of IDIG Registry). Setting alpha (probability of a type I error) equal to 0.05 and beta (probability of a type II error) equal to 90%, the required sample size was 74 patients for a two-sided test. To ensure the appropriate sample size, 82 patients were recruited to allow for drop outs.

Results obtained in the different treatment groups were analyzed blind by a team of statisticians. Differences in clinical remission proportions between patient groups were evaluated by the one-sided Fisher's exact probability test. For the analysis of the integrated measures of metabolic control (C-peptide, HbA_{1c} and insulin dose), an analysis of variance was done; for

measuring differences between groups at different time intervals, the Mann-Whitney U test was used.

For the analysis of antibody results, median antibody values in the two groups at each time point were compared using the Mann-Whitney U test, whereas proportions of patients with IA at each time point were compared using Fisher's exact probability test.

Results

Recruitment lasted 1 year. There were no significant differences between the two groups of patients in baseline clinical characteristics and metabolic control at the time of enrollment (Table 1). None of the patients suffered from any other autoimmune disease.

Dropouts. Only two patients withdrew from the study and this was because of poor compliance.

Metabolic data. Clinical remission was observed in one patient (lasting 3 months) in the oral insulin group and one patient (lasting 8 months) in the placebo group. Insulin requirement was significantly reduced in all patients after 3 months of treatment compared with the beginning of the trial but the patients treated with oral insulin and placebo did not differ in this respect (Table 2). The subcutaneous insulin dose required to obtain optimal metabolic control was similar in the two groups at 6, 9, and 12 months. Basal C-peptide secretion over 1 year of follow-up had a similar pattern in both groups of patients, with an initial increase (compared with diagnosis), followed by a steady decrease which was slightly more pronounced in the oral insulin group. When age at diagnosis was taken into account, insulin dose, HbA_{1c} values and C-peptide concentrations in patients older than 15 years ($n = 28$) were not different at the beginning of the trial between oral insulin ($n = 16$) and placebo-treated ($n = 12$) patients and did not change thereafter. In patients younger than 15 years ($n = 52$), C-peptide concentrations after 9 and 12 months tended to decline more in the oral insulin ($n = 28$) than in the placebo group ($n = 24$), although the difference between the two groups did not reach statistical significance (Fig. 1). Good metabolic control was achieved by all patients, as shown by the rapid decline in HbA_{1c} values after diagnosis, which persisted until the end of the study. Finally, no adverse effects were noted in patients receiving either oral insulin or placebo.

Insulin antibodies. Insulin antibodies were detectable at disease onset in 17 out of 27 (63%) patients receiving oral insulin and 8 out of 18 (44%) receiving placebo; the humoral response against insulin increased during the study in 23 of the patients receiving oral insulin and 15 of those treated with placebo. No dif-

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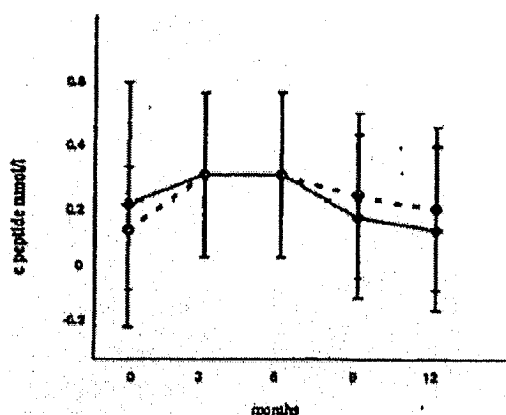


Fig. 1. Basal C-peptide concentration (mean \pm SD) in patients younger than 15 years treated with oral insulin ($n = 28$, —●—) and placebo ($n = 24$, - -●- -). Values were lower at 9 and 12 months in the patients treated with oral insulin, however they were not statistically different from those patients treated with placebo

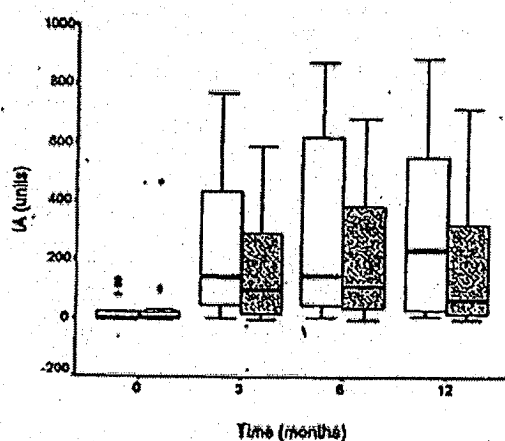


Fig. 2. Box-whisker plot of insulin antibodies measured at various time points in patients treated with oral insulin (filled boxes) or placebo (open boxes). Values between the two groups were not statistically different at any time point

ferences were observed between the two groups in the proportion of patients with IA or in the median insulin antibody concentrations at all time points, although a trend towards lower antibody concentrations was evident in the group treated with oral insulin after 3, 6 and 12 months of treatment (Fig. 2). When antibody results were analysed according to age at diagnosis (15 years), levels of IA after 3, 6 and 12 months were higher in younger patients ($n = 32$)

than in older subjects ($n = 13$) ($p < 0.02$ at all time points) but no significant differences were observed between patients treated with oral insulin and those treated with placebo when subdivided by age (data not shown).

Discussion

This double-blind trial with oral insulin in patients with recent-onset Type 1 diabetes was designed to assess whether the addition of oral insulin at the time of clinical diagnosis could maintain or even improve the residual beta-cell function which is usually detectable in these patients. Oral insulin had no effect on residual beta-cell function, as assessed by C-peptide secretion. Furthermore, patients treated with oral insulin who were younger than 15 years at diagnosis showed a tendency for a more pronounced decline of basal C-peptide concentrations 9 and 12 months after diagnosis compared with patients matched with them for age but treated with placebo, although this difference was not statistically significant. We did not measure stimulated C peptide but limited the investigation to baseline C peptide concentrations. These were measured under strict and controlled conditions of fasting blood glucose less than 180 mg/kg at the time of sampling (if blood glucose concentration was higher sampling for C peptide was postponed). The night before the test patients were also advised to have a light meal and avoid any unnecessary stress. In the light of the results of baseline C-peptide concentrations it is doubtful that oral insulin had an effect on those of stimulated C-peptide. Older patients also did not benefit from the addition of oral insulin: thus, this antigen-based therapy, which is supposed to induce tolerance to a key antigen (e.g. insulin) in Type 1 diabetes, seems to be ineffective (at least at the doses used in this trial) in protecting residual beta-cell function in patients with recently diagnosed disease.

There are a number of possibilities to explain these findings. One is that the oral insulin daily dose used in this trial (5 mg) was not sufficient. Several experimental data have indicated that the dose of antigen is a critical factor for tolerance induction in autoimmune diseases [13]. A similar trial in France, in which two doses of oral insulin (2.5 mg and 7.5 mg) were used and preliminary data presented in abstract form did not show any effect [14]. As the addition of oral insulin does not influence metabolic control, higher doses of oral insulin should possibly be tested. The use of an adjuvant carrier to increase the tolerogenic capacity of insulin is also worth consideration for tolerance induction, with implications for clinical use [15].

Another possibility is that at the time of clinical diagnosis of Type 1 diabetes residual beta-cell mass is so small that the efficacy of this treatment cannot be de-

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tested. This might be different in pre-Type 1 diabetes, in which beta-cell mass is almost unaffected and the spreading of the autoimmune response to a number of other antigens, which generally amplifies the rate of beta-cell destruction, is still limited. In such a case tolerance induction might still be possible with insulin. It is, however, of concern that in our trial treatment with oral insulin seemed to accelerate the decline of beta-cell function, at least in the very young subjects, because insulin is considered to be the major target of the autoimmune attack against beta cells, especially in young patients. In other autoimmune condition(s) the addition of oral antigens has induced an accelerating effect on disease progression [16]. A reasonable concern is that if oral insulin has no or negative effects on the natural course of beta-cell destruction in the first year after diagnosis it might have similar effects when given before the onset of overt hyperglycaemia. The trend towards lower IA responses observed in patients treated with oral insulin might reflect a modulation of the response induced against exogenous insulin. The reduction was, however, not statistically significant, so it is difficult to draw any definitive conclusion on the effect of oral insulin given at disease onset in terms of modulation of antigen-specific immune reactivity.

All these concerns apply to the prevention trials designed to test whether intervention during the prodromal period of Type 1 diabetes can delay its clinical onset. Specifically, the objective of the DPT-1 is to determine whether antigen-based therapies (e.g. insulin) in non-diabetic relatives of patients with Type 1 diabetes can delay the development of overt clinical disease [1]. Based on the results of our trial attention should be paid to the subjects in the oral treatment group of DPT-1 trial to find out whether oral insulin affects C-peptide secretion or insulin antibody concentrations.

In conclusion, the addition of 5 mg daily of oral insulin to regular subcutaneous insulin therapy has no effect on residual beta-cell function in patients with recent-onset Type 1 diabetes and does not modify the humoral immune response against the hormone. These results have important implications for current thoughts in designing strategies for preventing Type 1 diabetes.

Acknowledgements. The work was supported by grants from The Italian Office for University and Scientific and Technological Research (MURST) and The International Centre for the Study of Diabetes. Insulin for oral treatment was kindly donated by Eli Lilly. The group in Palermo was supported by a grant from the Italian Society of Diabetes (C. Giordano). We also acknowledge Dr E. Bonifacio and Prof E. Bosi for their comments and Miss C. Devlin for helping to prepare the final version of this paper.

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EXHIBIT 8

Effects of Oral Insulin in Relatives of Patients With Type 1 Diabetes

The Diabetes Prevention Trial—Type 1

THE DIABETES PREVENTION TRIAL—TYPE 1
STUDY GROUP

OBJECTIVE— This randomized, double-masked, placebo-controlled clinical trial tested whether oral insulin administration could delay or prevent type 1 diabetes in nondiabetic relatives at risk for diabetes.

RESEARCH DESIGN AND METHODS— We screened 103,391 first- and second-degree relatives of patients with type 1 diabetes and analyzed 97,273 samples for islet cell antibodies. A total of 3,483 were antibody positive; 2,523 underwent genetic, immunological, and metabolic staging to quantify risk of developing diabetes; 388 had a 5-year risk projection of 26–50%; and 372 (median age 10.25 years) were randomly assigned to oral insulin (7.5 mg/day) or placebo. Oral glucose tolerance tests were performed every 6 months. The median follow-up was 4.3 years, and the primary end point was diagnosis of diabetes.

RESULTS— Diabetes was diagnosed in 44 oral insulin and 53 placebo subjects. Annualized rate of diabetes was similar in both groups: 6.4% with oral insulin and 8.2% with placebo (hazard ratio 0.764, $P = 0.189$). In a hypothesis-generating analysis of a subgroup with insulin autoantibody (IAA) levels confirmed (on two occasions) ≥ 80 nU/ml ($n = 263$), there was the suggestion of benefit: annualized diabetes rate 6.2% with oral insulin and 10.4% with placebo (0.566, $P = 0.015$).

CONCLUSIONS— It is possible to identify individuals at high risk for type 1 diabetes and to enroll them in a large, multisite, randomized, controlled clinical trial. However, oral insulin did not delay or prevent type 1 diabetes. Further studies are needed to explore the potential role of oral insulin in delaying diabetes in relatives similar to those in the subgroup with higher IAA levels.

Diabetes Care 28:000–000, 2005

Article

The Diabetes Prevention Trial—Type 1 (DPT-1) was a randomized controlled clinical initiative designed to determine whether it is possible to prevent or delay the onset of overt diabetes in relatives of patients with type 1 diabetes. DPT-1 included two separate trials. Relatives were screened for islet cell antibodies (ICAs), and those who were positive underwent further testing to assess pro-

jected 5-year risk of diabetes. Earlier we reported the results of the DPT-1 paternal insulin trial, conducted in relatives with $>50\%$ projected 5-year risk of diabetes (1). This article reports the results of the DPT-1 oral insulin trial in relatives with a projected 5-year risk of diabetes of 26–50%. In both trials, relatives were studied because of their 10- to 20-fold increased risk compared with the general population (2,3).

Type 1 diabetes is a consequence of immune-mediated destruction of insulin-secreting pancreatic islet β -cells (4). A number of studies have suggested that oral administration of autoantigens induces protective immunity that has the potential to downregulate ongoing destructive immune reactions (5–7). Peptides derived from an orally administered antigen encounter the mucosal gut-associated lymphoid tissue, which serves both to protect the host from ingested pathogens and to prevent the host from reacting to ingested proteins. The concept is that low doses of orally administered autoantigens suppress autoimmunity by inducing antigen-specific regulatory T-cells in the gut, which act by releasing inhibitory cytokines at the target organ (5–7). In the mid-1990s, the concept of oral antigen administration was quite popular, and studies were initiated in a number of human autoimmune diseases. In the nonobese diabetic mouse model of type 1 diabetes, oral administration of insulin to young, pre-diabetic mice inhibits their development of type 1 diabetes (8–13). Oral insulin also prevented diabetes and even reversed hyperglycemia in a transgenic mouse model of virus-induced diabetes (14). The results in these animal models suggested that oral insulin could attenuate pancreatic islet autoimmunity, leading to a delay in the onset of the disease, and was the impetus to conduct the DPT-1 oral insulin trial. Moreover, the breakdown of insulin into smaller peptides in the gastrointestinal tract would avoid any hypoglycemic effects of insulin,

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This manuscript was prepared by a writing and review committee consisting of Jay S. Skyler, MD; Jeffrey P. Krischer, PhD; Joseph Wolfsdorf, MD; Catherine Cowie, PhD; Jerry P. Palmer, MD; Carla Greenbaum, MD; David Cuthbertson, MS; Lisa E. Rafkin-Mervis, MS; H. Peter Chase, MD; and Ellen Leschek, MD.

Jay S. Skyler, MD (DPT-1 Study Chairman), takes full responsibility for the analysis and integrity of the data and the writing of the manuscript.

Additional information for this article can be found in an online appendix at <http://care.diabetesjournals.org>.

Abbreviations: DPT-1, Diabetes Prevention Trial—Type 1; FPG, fasting plasma glucose; FPIR, first-phase insulin response; IAA, insulin autoantibody; ICA, islet cell antibody; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; OGTT, oral glucose tolerance.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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an additional potential benefit for testing oral insulin.

RESEARCH DESIGN AND METHODS

The study was divided into three parts: screening, staging, and intervention (1). Participants were recruited from study clinics and through media campaigns.

Screening

First-degree (ages 3–45 years) and second-degree (ages 3–20 years) relatives of patients with type 1 diabetes were screened for ICAs. Those with ICA titer ≥ 10 Juvenile Diabetes Foundation units were invited to have staging evaluations.

Staging

Staging confirmed ICA positivity, measured insulin autoantibody (IAA) status, assessed first-phase insulin response (FPIR) to intravenous glucose, assessed oral glucose tolerance (OGT), and determined presence or absence of HLA-DQA1*0102/DQB1*0602 (a protective haplotype that excluded subjects from participation) (15,16). Relatives who were ICA⁺ and IAA⁺ and with FPIR above threshold (defined below) and normal glucose tolerance were projected to have a 5-year risk of 26–50% ("intermediate risk") and were eligible for the oral insulin trial. Those identified as having a 5-year risk of $\geq 50\%$ ("high risk") were eligible for the parenteral insulin trial previously reported (1). The original protocol had an entry criterion of confirmed (on two occasions) IAA level > 5 SD above the mean of the normal reference range (i.e., ≥ 80 nU/ml). In October 1997, after review of data from natural history studies suggesting that a sufficient cutoff was > 3 SD above the mean of the reference range, to enhance enrollment the entry criterion was changed to that level (i.e., IAA ≥ 39 nU/ml).

Intervention

The study was a double-masked, placebo-controlled, randomized clinical trial, in which participants were assigned to receive capsules of either oral insulin, 7.5 mg of recombinant human insulin crystals (Eli Lilly, Indianapolis, IN), or matched placebo. Capsules were prepared with methylcellulose filler at a compounding pharmacy (Belmar Pharmacy, Lakewood, CO). Masked bottles of oral insulin or placebo were shipped to clinical

sites from a research pharmacy (Moffitt Cancer Center, Tampa, FL). Subjects consumed the capsule as a single daily dose before breakfast each day, either by taking the capsule or, if the subject could not swallow capsules, sprinkling its contents in juice or on food. Randomization used a central automated system, stratified by clinical center, using random variable block sizes.

Study sites

Study coordination, laboratory tests, and data management were done centrally. Protocols were approved by institutional review boards at all participating locations across the U.S. and Canada, including 91 sites conducting the intervention. Participants (and/or their parents) provided separate written consent for each part, screening, staging, and intervention, and yearly thereafter for continuation in the study.

Role of the funding source

Representatives from the sponsoring institutes of the National Institutes of Health (National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Allergy and Infectious Diseases, and National Institute of Child Health and Human Development) served on the Steering Committee and virtually all of the study group committees and were full participants on their committees, which were involved in all aspects of protocol design, data analysis, and preparation of the manuscript. The other funding sources provided only resources and were not involved in the study per se.

Follow-up assessments

Participants were seen every 6 months, and at those visits an OGT test was performed to assess glycemic status, the primary study end point. An intravenous glucose tolerance test was performed at baseline, annually thereafter, and at study end. Mixed-meal tolerance tests were performed at baseline, after 3 years, and at study end.

Participants checked blood glucose if they experienced symptoms of hypoglycemia. Presumed hypoglycemia (without measurement of glucose) was defined as typical symptoms that promptly resolved with food intake. Definite hypoglycemia was defined as blood glucose < 2.8 mmol/l (50 mg/dl) measured at the time of symptoms. Severe hypoglycemia was

defined as loss of consciousness, convulsion, stupor, or hypoglycemia requiring assistance of another person or treatment with intravenous glucose or subcutaneous glucagon. Chemical hypoglycemia was defined by five-point (before breakfast, before lunch, before supper, 2 h after supper, 3:00 A.M.) home capillary blood glucose profiles obtained quarterly, if two of these glucose values were < 2.8 mmol/l (< 50 mg/dl).

Tolerance test procedures

Tolerance tests were performed after an overnight fast. Samples were drawn through a temporary indwelling intravenous catheter. Intravenous glucose tolerance tests were performed as described (17,18). Insulin values at 1 and 3 min were added to determine FPIR. FPIR was above threshold if ≥ 10 th percentile for siblings, offspring, and second-degree relatives (≥ 100 μ U/ml if age ≥ 8 years; ≥ 60 μ U/ml if age < 8 years) and ≥ 1 st percentile for parents (≥ 60 μ U/ml). FPIR above threshold was required for eligibility.

For the oral glucose tolerance test, the oral glucose (Sundex, Fisher) dose was 1.75 g/kg (maximum 75 g). Plasma glucose values were interpreted according to American Diabetes Association guidelines (19): fasting plasma glucose (FPG) ≥ 7.0 mmol/l (≥ 126 mg/dl) or 120-min glucose ≥ 11.1 mmol/l (≥ 200 mg/dl) was considered diagnostic of diabetes; FPG 6.1–6.9 mmol/l (110–125 mg/dl) signified impaired fasting glucose (IFG); 120-min glucose 7.8–11.1 mmol/l (140–199 mg/dl) signified impaired glucose tolerance (IGT). If a 30-, 60-, or 90-min level was > 11.1 mmol/l (> 200 mg/dl) but FPG and 120-min levels were below threshold for IFG and IGT, this was noted as indeterminate glucose tolerance. A normal OGT during staging was required for eligibility. Diagnosis of diabetes required confirmation on a subsequent day by OGT, elevated fasting plasma glucose, or random plasma glucose ≥ 11.1 mmol/l (≥ 200 mg/dl) accompanied by symptoms of polyuria, polydipsia, and/or weight loss (19).

For the mixed-meal tolerance test, a liquid formula meal was consumed (Sustacal/Boost, Mead Johnson Nutritionals; 6 kcal/kg body weight, maximum 360 kcal).

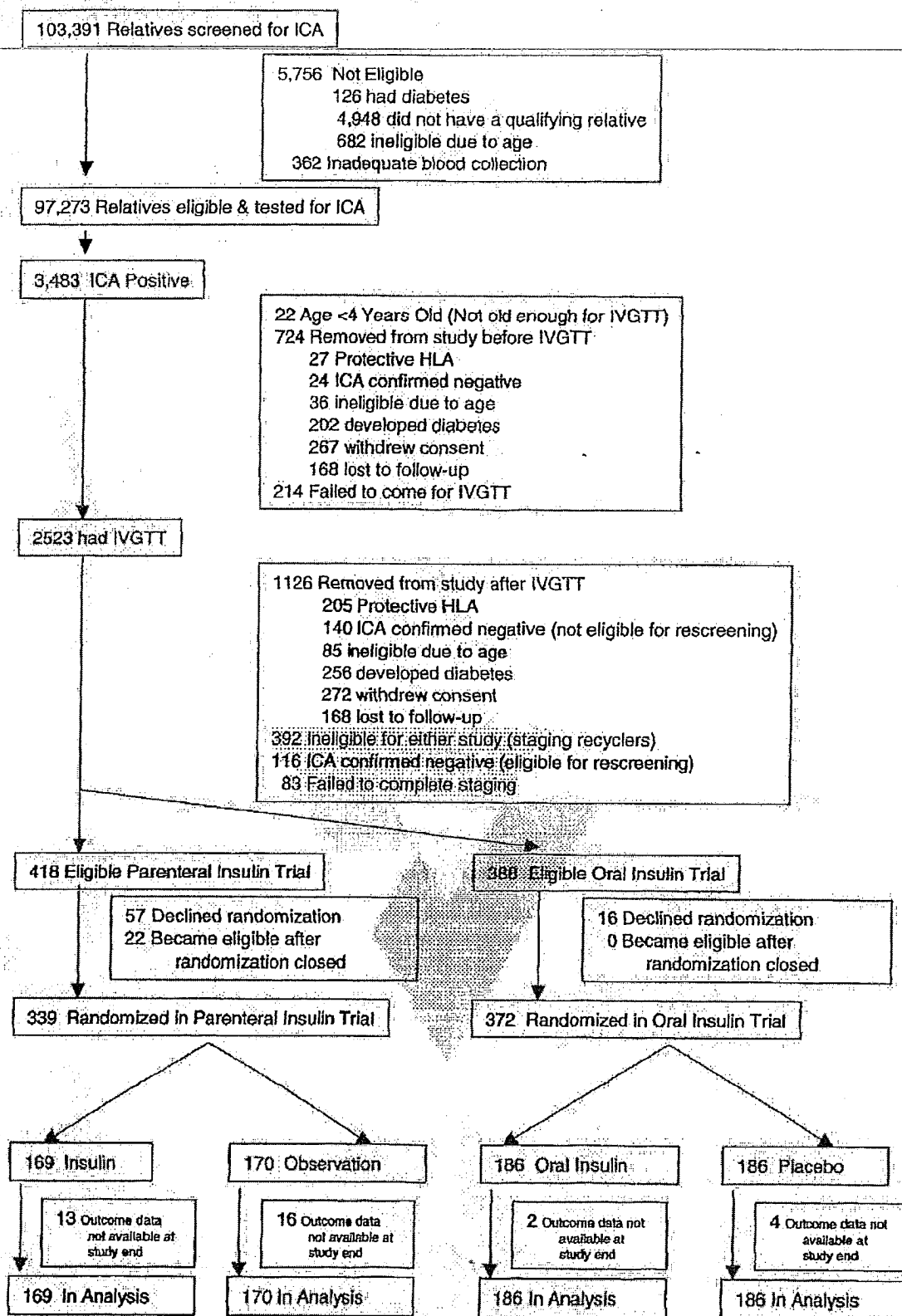


Figure 1—Flow diagram of all subjects recruited to trial. IVGTT, intravenous glucose tolerance test.

Table 1—Baseline characteristics of randomly assigned subjects

	Oral insulin group	Placebo group	P value
n	186	186	
Median age	11.0 (7–14)	9.5 (7–14)	0.3569
Average FPIR (μ U/ml)	161.6 \pm 72.4	158.9 \pm 99.2	0.7672
Race			0.2807
White	164 (88.1)	163 (87.6)	
African American	5 (2.6)	2 (1.0)	
Hispanic	8 (4.3)	14 (7.5)	
Other	9 (4.7)	7 (3.7)	
Sex			0.1381
Male	119 (63.9)	105 (56.4)	
Female	67 (36.0)	81 (43.5)	
Relationship to index patient with diabetes			0.6552
Sibling	112 (60.2)	108 (58.0)	
Offspring	49 (26.3)	53 (28.4)	
Parent	11 (5.9)	7 (3.7)	
Second degree	14 (7.5)	18 (9.6)	
Antibody levels			
Median ICAs (JDF units)	80 (403–20)	80 (40–160)	0.9253
Mean IAAs (nU/ml)	382 \pm 555	346 \pm 436	0.4910
GAD antibodies			0.2908
Positive	144 (77.8)	136 (56.4)	
Negative	41 (22.1)	50 (43.5)	
ICA-512 antibodies			0.9567
Positive	97 (52.4)	97 (52.1)	
Negative	88 (47.5)	89 (47.8)	
Micro IAA			0.0551
Positive	39 (29.3)	28 (19.4)	
Negative	94 (70.6)	116 (80.5)	
HbA _{1c} (%)	5.35 \pm 0.39	5.33 \pm 0.34	0.5949
C-peptide area under curve			
During intravenous glucose tolerance test	34.8 (15.6)	35.1 (16.7)	0.8800
During oral glucose tolerance test	502.5 (201.1)	502.1 (207.2)	0.9858
During mixed meal tolerance test	383.1 (172.4)	381.0 (383.8)	0.9102

Data are means \pm SD, n (%), or mean (interquartile range).

Laboratory measures

All assays were performed as previously described (1), including ICA (indirect immunofluorescence), IAA (competitive fluid-phase radioassay), plasma glucose (glucose oxidase method), insulin (radioimmunoassay), C-peptide (radioimmunoassay), and HLA-DQ typing (PCR using sequence-specific probes).

Statistical methods

The trial was designed assuming a 5-year cumulative diabetes incidence of 26–50% (annual hazard rate 6%), 80% power to detect a 50% reduction in incidence in the oral insulin group, and $\alpha = 0.05$ (two-tailed). The oral insulin trial was designed to accrue subjects for 4 years with 2 years of follow-up and an annual rate of loss to follow-up of 10%, yielding an es-

timated average planned duration of treatment of 2.8 years with a projected 70 events occurring. The original projection was for a 4-year accrual period and sample size of 490 subjects in the oral insulin trial.

Variables not normally distributed were log-transformed for analysis and back-transformed for presentation. Data were analyzed according to the intention-to-treat principle. Kaplan-Meier life tables were constructed and compared by the log-rank χ^2 statistic. Categorical variables were compared by Pearson's χ^2 test or Fisher's exact test. Differences in means were tested using ANOVA. Tests of significance were two-tailed. Statistical analyses were performed using SAS software. Data on safety and efficacy were evaluated twice yearly by an independent Data

Safety Monitoring Board, with predefined stopping rules.

RESULTS—Screening began on 15 February 1994, and the first subject in this protocol was randomly assigned on 10 September 1996. The actual enrollment period was 6.1 years. By the time randomization was completed (31 October 2002), screening samples for ICA had been obtained from 103,391 relatives. Of these, 97,634 were eligible for further study. Ineligible samples came from individuals without an identified relative with diabetes or not in the age range defined by the protocol. By the end of enrollment, 97,273 samples were analyzed for ICA and 3,483 (3.58%) relatives were ICA positive. Of these, 458 (13.1% of ICA⁺ individuals) were excluded before randomization because they already had diabetes. A total of 2,523 (72.4% of ICA⁺ individuals) underwent staging. There were 1,844 relatives with intravenous glucose tolerance FPIR above threshold. As staging continued, a total of 388 relatives were classified as intermediate risk and eligible for randomization; of these, 372 were randomized (97% of eligible subjects), 186 to each study arm (Fig. 1). Table 1 shows baseline characteristics; there were no statistically significant differences between treatment groups. Online appendix Fig. 1 (available at <http://care.diabetesjournals.org>) shows the frequency distribution of age at randomization by treatment arm.

Participants were followed for a median of 1,582 days (4.3 years; interquartile range 928–1988). Annual rate of loss to follow-up was 0.2%, less than anticipated in the protocol (10%). Annual non-compliance rate was 3.7% in the oral insulin group and 6.6% in the placebo group, with noncompliance being failure to attend for scheduled tests and/or failure to take study medication.

Final primary end point data were available for 98.4% of subjects randomized. Diabetes was diagnosed in 97 participants—44 in the oral insulin group and 53 in the placebo group. The majority (72%) of participants were asymptomatic at the time of diagnosis and/or were detected by study OGT tests. The proportion of participants who developed diabetes, averaged annually over follow-up, was 6.4% per year in the oral insulin group and 8.2% per year in the placebo group. Cumulative incidence of diabetes

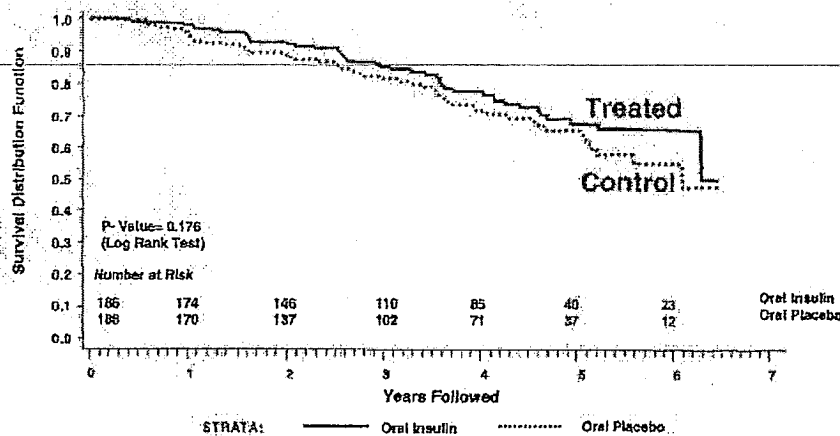


Figure 2—Kaplan-Meier curves showing the proportion of subjects without diabetes during the trial, by treatment assignment. The number of subjects at risk in each group at each year of follow-up is enumerated at the bottom of the figure. The log-rank test was used for comparison between the groups, with the P values as indicated.

was similar in both groups (hazard ratio 0.764, 95% CI 0.511–1.142, $P = 0.189$) (Fig. 2).

Progression to suspected diabetes (diabetes on one occasion not subsequently confirmed) and progression to first abnormal OGT test were examined separately (online appendix Fig. 2A and B). No treatment differences were found. Time to FPIR below threshold was also examined and again no difference was found (online appendix Fig. 2C).

Insulin secretion was examined before diagnosis of diabetes by assessing the C-peptide response during OGT and mixed-meal tolerance tests. There was no

difference between groups for peak C-peptide value or area under the curve. Online appendix Fig. 3 shows area under the curve C-peptide values during OGT tests.

There was no difference in glycemia between groups in the intention-to-treat analysis. A secondary regression analysis revealed that, compared with those who did not develop diabetes, subjects who progressed to diabetes had a slight progressive increase in both HbA_{1c} ($P < 0.001$) and area under the curve glucose on serial OGT tests ($P < 0.001$).

There were no serious adverse events and no differences between groups in fre-

quency of adverse events. Rate of chemical hypoglycemia, assessed without ascertainment bias, was 4.4 per 100 patient-years in the oral insulin group and 3.4 per 100 patient-years in the placebo group ($P = 0.387$). There were no reported episodes of severe hypoglycemia.

As noted, the initial entry criterion for IAA was a level ≥ 80 nU/ml, which was subsequently changed to a level ≥ 39 nU/ml. There was the suggestion of an increased rate of progression to diabetes in subjects with IAA values ≥ 80 nU/ml (confirmed on two occasions; $n = 263$) compared with those with IAA values not confirmed ≥ 80 nU/ml (in which at least one or both measurements were 39–79 nU/ml; $n = 109$; $P = 0.052$) (Fig. 3). Table 2 shows baseline characteristics in those two cohorts; subjects with confirmed IAA values ≥ 80 nU/ml were younger and more likely to be male and had higher ICA titers, higher frequency of other autoantibodies, and lower levels of C-peptide. All of these characteristics are consistent with higher risk of diabetes.

Among participants with confirmed IAA ≥ 80 nU/ml ($n = 263$), the proportion who developed diabetes was 6.2% per year in the oral insulin group and 10.4% per year in the placebo group, averaged annually over follow-up (hazard ratio 0.566, 95% CI 0.361–0.888; $P = 0.015$) (Fig. 4). From the data, the delay in diabetes, calculated from median survival times, is projected as 4.5 years. Online appendix Table 1 shows baseline characteristics of this cohort, except for greater proportion of males in the oral insulin group, there were no statistically significant differences between treatment groups.

In contrast, among participants not confirmed as IAA ≥ 80 nU/ml ($n = 109$), the proportion who developed diabetes was 6.9% per year in the oral insulin group and 2.7% per year in the placebo group, averaged annually over follow-up (hazard ratio 2.702, 95% CI 0.949–7.694; $P = 0.079$; online appendix Fig. 4). Online appendix Table 2 shows baseline characteristics; there were no statistically significant differences between the oral insulin and placebo groups.

In an analysis confined to subjects randomized before the change in IAA criterion on 31 October 1997 ($n = 106$), all of whom had confirmed IAA ≥ 80 nU/ml, the proportion who developed diabetes was 6.4% per year in the oral insulin

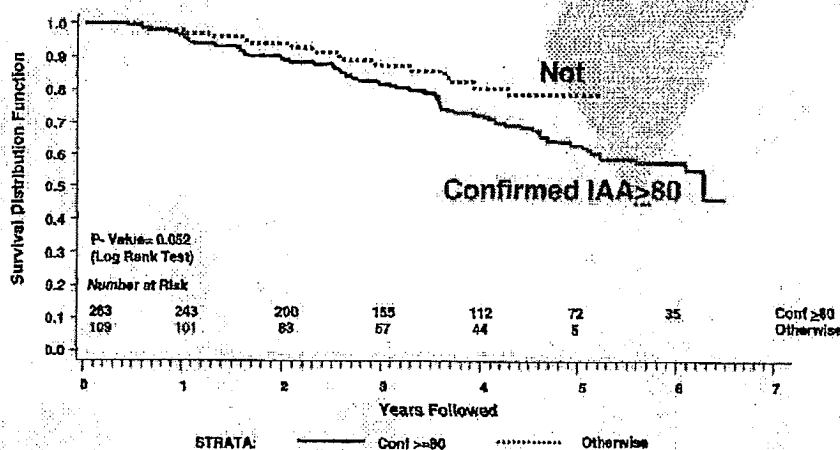


Figure 3—Kaplan-Meier curves showing the proportion of subjects without diabetes during the trial by baseline IAA level (confirmed value ≥ 80 nU/ml vs. at least one value 39–79). The number of subjects at risk in each group at each year of follow-up is enumerated at the bottom of the figure. The log-rank test was used for comparison between the groups, with the P values as indicated.

Table 2—Baseline characteristics of subjects by IAA status

	Not confirmed IAA ≥ 80	Confirmed IAA ≥ 80	P value
n	109	263	
Median age	13.0 (9–18)	9.0 (6–12)	0.0000
Average FPIR ($\mu\text{U/ml}$) (SD)	172.1 \pm 73.1	155.3 \pm 91.4	0.0878
Race			0.9246
White	96 (88.0)	231 (87.8)	
African American	2 (1.8)	5 (1.9)	
Hispanic	7 (6.4)	15 (5.7)	
Other	4 (3.6)	12 (4.5)	
Sex			0.0445
Male	57 (52.2)	167 (63.5)	
Female	52 (47.7)	96 (36.5)	
Relationship to index patient with diabetes			0.1649
Sibling	58 (53.2)	162 (61.6)	
Offspring	31 (28.4)	71 (27.0)	
Parent	9 (8.2)	9 (3.4)	
Second degree	11 (10.0)	21 (7.9)	
Antibody levels			
Median ICAs (IDF units)	40 (20–160)	80 (40–320)	0.0001
Mean IAAs (nU/ml)	72.0 \pm 72.3	485.2 \pm 547.5	0.0000
GAD antibodies			0.0461
Positive	74 (68.5)	206 (78.3)	
Negative	34 (31.4)	57 (21.6)	
ICA-512 antibodies			0.0043
Positive	44 (40.7)	150 (57.0)	
Negative	64 (59.2)	113 (42.9)	
Micro IAA			0.0000
Positive	4 (5.0)	63 (31.9)	
Negative	76 (95.0)	134 (68.0)	
HbA _{1c} (%)	5.33 \pm 0.37	5.35 \pm 0.36	0.6112
C-peptide area under curve			
During intravenous glucose tolerance test	40.1 (16.7)	32.8 (15.4)	0.0001
During oral glucose tolerance test	563.9 (225.0)	476.6 (189.1)	0.0002
During mixed-meal tolerance test	443.2 (183.3)	365.2 (169.5)	0.0000

Data are means \pm SD, n (%), or mean (interquartile range).

group and 11.3% per year in the placebo group, averaged annually over follow-up (hazard ratio 0.539; 95% CI 0.298–0.976; $P = 0.035$) (Fig. 5). From the data, the delay in diabetes, calculated from median survival times, is projected as 4.8 years. Online appendix Table 3 shows baseline characteristics; there were no statistically significant differences by treatment.

CONCLUSIONS—Oral insulin has been used in three studies to test the concept of oral antigen administration in an effort to preserve pancreatic islet β -cell function in newly diagnosed type 1 diabetes (20–22). All three trials failed to show a consistent beneficial effect. Likewise, in BB rats, oral insulin not only failed to prevent type 1 diabetes (23) but,

when administered with an adjuvant, actually accelerated the development of diabetes (24). This finding is in stark contrast with the beneficial effects of oral insulin observed in the nonobese diabetic mouse (8–13) and in a transgenic mouse model of virus-induced diabetes (14). Oral antigen administration had only small and inconsistent benefits in clinical trials in multiple sclerosis and rheumatoid arthritis, despite success in animal models of those autoimmune diseases.

Unfortunately, in the primary analysis of relatives selected and randomized in DPT-1, oral insulin did not delay or prevent development of diabetes. There was greater variability in the IAA assay for values 39–79 nU/ml than for values ≥ 80 nU/ml, particularly in confirmation of a

positive result (98.7% overall confirmation for values ≥ 80 nU/ml compared with 70.6% for values 39–79 nU/ml). This prompted comparison of the rate of evolution of diabetes by entry IAA level (Fig. 3). The cohort with confirmed IAA ≥ 80 nU/ml (the original entry IAA criterion) progressed to diabetes at a faster rate than those subjects who did not have confirmed IAA ≥ 80 nU/ml. In addition, those with confirmed IAA ≥ 80 nU/ml had other risk characteristics that suggest more rapid evolution to diabetes, including younger age, greater likelihood of having other antibodies, and greater loss of β -cell function (lower levels of plasma C-peptide in response to several provocative challenges).

We then examined the effects of intervention in each of these two subgroups. The group with confirmed IAA ≥ 80 nU/ml showed a beneficial effect of oral insulin, whereas the group who did not have confirmed IAA ≥ 80 nU/ml showed a trend suggesting a detrimental effect of oral insulin. This group also had a much lower overall rate of development of diabetes. Thus, the significance of this finding is unclear but is reminiscent of the adjuvant induced acceleration of diabetes observed in the BB rat (24).

To gain further insight into the impact of the change that was made in the entry IAA criterion, we performed an analysis confined to subjects randomized before the change in IAA criterion (31 October 1997), all of whom had confirmed IAA ≥ 80 nU/ml. In this analysis, the results were comparable to those seen in all subjects with confirmed IAA ≥ 80 nU/ml. There is an obvious lesson for clinical trialists not to tamper with the trial design because enrollment is lagging. One might hypothesize that there might have been a clear beneficial result in the overall trial if the IAA entry criterion had not been changed. However, because none of these subgroup analyses were prespecified, the results suggesting a potential beneficial effect in the subgroup with baseline-confirmed IAA ≥ 80 nU/ml (either all subjects or those enrolled before the protocol change) can only be deemed hypothesis-generating and not a positive outcome. As a consequence, the successor study group to DPT-1, the Type 1 Diabetes TrialNet clinical trials network, is contemplating a confirmatory study to explore the potential role of oral insulin in delaying or preventing type 1 diabetes in relatives found

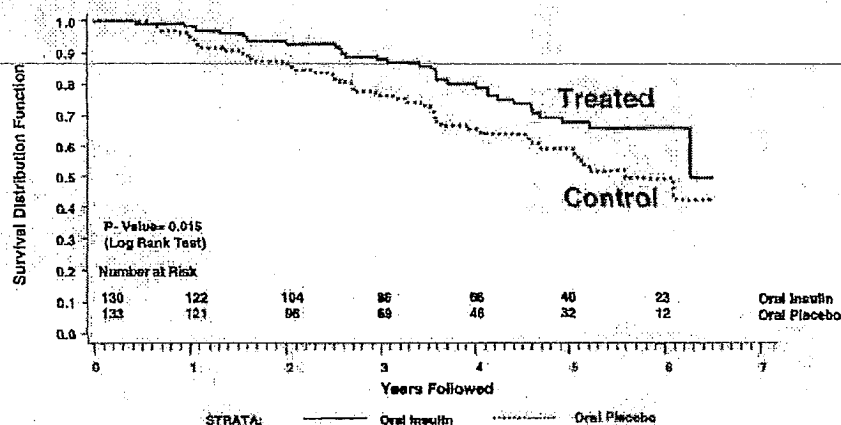


Figure 4—Kaplan-Meier curves showing the proportion of subjects without diabetes during the trial by treatment assignment for subjects with baseline confirmed IAA ≥ 80 nU/ml. The number of subjects at risk in each group at each year of follow-up is enumerated at the bottom of the figure. The log-rank test was used for comparison between the groups, with the P values as indicated.

to be at risk for diabetes with IAA levels similar to those in the DPT-1 subgroup.

There are several possible explanations for failure to demonstrate efficacy in the primary analysis. One is that oral insulin has no effect. Another is that inclusion of subjects with variable and lower risk of diabetes (those with IAA 39–79 nU/ml) may have masked a treatment effect or that in some of these subjects diabetes may have been accelerated. A third possibility is that the dose used in this study was unable to sufficiently stimulate the immune system, but this is difficult to test because we have no established immunologic biomarkers of disease progres-

sion. Perhaps if an adjuvant had been used, some effect would have been more evident. In animal models that tested oral insulin, heterologous (either porcine or human) insulin was used. It is possible that homologous insulin, as used here, may have failed to elicit a protective immunologic response. Lastly, the timing of our intervention may have been incorrect. Although there has been speculation that once the immunologic markers used to detect relatives at increased risk for type 1 diabetes are detectable then the destructive immune response may be irreversible by an antigen-based therapy, it is of interest that the subgroup who may have had

some benefit of therapy had evidence of being further along in the disease process (higher antibody levels, greater number of antibodies, and lower levels of C-peptide).

The parameters used to predict development of diabetes in relatives of individuals with diabetes were accurate. Risk was projected to be 26–50%, whereas actual risk was 35% over 5 years. Similarly, in our previously reported parenteral insulin trial, 5-year risk was projected to be >50% and actual risk was 65% (1). The ability to quantify risk in relatives of patients with type 1 diabetes and to randomly assign those relatives in controlled clinical trials permits the design of studies that will ultimately lead to determination of whether the type 1 diabetes disease process can be altered in human beings to delay or prevent the development of clinical diabetes.

Three large randomized controlled trials designed to delay or prevent type 1 diabetes—the two DPT-1 trials and the European Diabetes Nicotinamide Intervention Trial (25)—have failed to demonstrate a treatment effect. It should be noted that of the myriad of interventions that had shown preclinical efficacy, both DPT-1 and the European Diabetes Nicotinamide Intervention Trial chose to use interventions with low toxicity in their attempts to interdict the type 1 diabetes disease process. Thus, it should not be concluded that it is impossible to delay or prevent type 1 diabetes; rather, it may require testing of more potent interventions or combinations of therapies, guided by better understanding of the immunopathogenesis of the disease, to demonstrate attenuation or amelioration of the destructive immune process leading to type 1 diabetes.

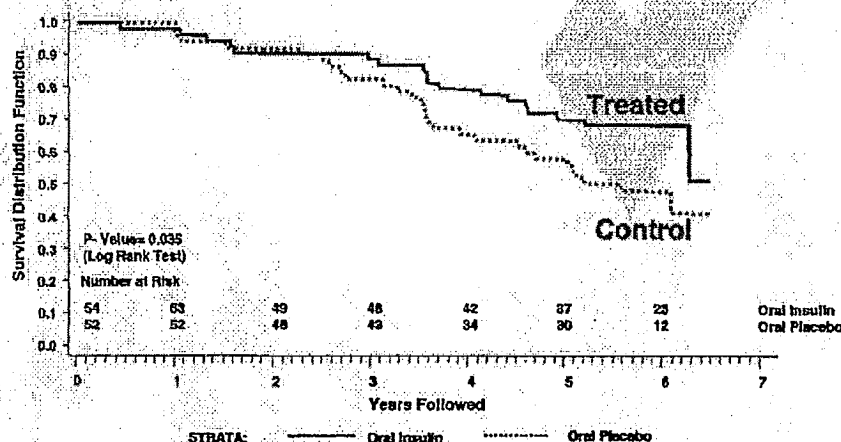


Figure 5—Kaplan-Meier curves showing the proportion of subjects without diabetes during the trial by treatment assignment for subjects enrolled before protocol change in entry criterion. The number of subjects at risk in each group at each year of follow-up is enumerated at the bottom of the figure. The log-rank test was used for comparison between the groups, with the P values as indicated.

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and Company, Bayer Corporation, Becton Dickinson and Company, International Technidyne Corporation, LifeScan, Inc., Mead Johnson Nutritionals Division of Bristol-Myers Squibb, Medisense Division of Abbott Laboratories, MiniMed Inc., and Roche Diagnostics.

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George S. Eisenbarth, MD, PhD, a member of the DPT-1 Steering Committee, is an inventor of a patent for the use of oral insulin in inducing immunological tolerance in the prevention or treatment of type 1 diabetes.

Parts of this study were presented at the annual meeting of the ADA, New Orleans, Louisiana, 13–17 June 2003, and at the Immunology of Diabetes Society meeting, Cambridge, U.K., 28–31 March 2004.

APPENDIX

DPT-1 Steering Committee

Jay S. Skyler, MD (University of Miami) (Chair), David Brown, MD (University of Minnesota), H. Peter Chase, MD (Barbara Davis Center for Childhood Diabetes, University of Colorado), Elaine Collier, MD (NIAID), Catherine Cowie, PhD (NIDDK), George S. Eisenbarth, MD (Barbara Davis Center for Childhood Diabetes, University of Colorado), Judith Fradkin, MD (NIDDK), Gilman Grave, MD (NICHD), Carla Greenbaum, MD (Benaroya Research Institute, Seattle), Richard A. Jackson, MD (Joslin Diabetes Center), Francine R. Kaufman, MD (Children's Hospital Los Angeles), Jeffrey P. Krischer, PhD (University of South Florida), Jennifer B. Marks, MD (University of Miami), Jerry P. Palmer, MD (University of Washington), Alyne Ricker, MD (Children's Hospital, Boston), Desmond A. Schatz, MD (University of Florida), Darrell Wilson, MD (Stanford University), William E. Winter, MD (University of Florida), Joseph Wolfsdorf, MD (Children's Hospital, Boston), Adina Zeidler, MD (University of Southern California). Previous members were Howard Dickler, MD, Richard C. Eastman, MD, Noel K. MacLaren, MD, John I. Malone, MD, and R. Paul Robertson, MD.

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dith Fradkin, MD, George S. Eisenbarth, MD, PhD, Carla Greenbaum, MD, Kevan Herold, MD (Columbia University), Francine R. Kaufman, MD, Jeffrey P. Krischer, PhD, Jennifer B. Marks, MD, Lisa E. Rafkin-Mervis, MS, Desmond A. Schatz, MD, and Jay S. Skyler, MD.

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DPT-1 Data Safety and Quality Monitoring Committee

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A complete listing of the DPT-1 Study Group appears in the online appendix. The DPT-1 Protocol and the DPT-1 Manual of Operations are available from the DPT-1 Operations Coordinating Center, University of Miami, 1450 NW 10th Ave., Suite 3054, Miami, FL 33136.

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EXHIBIT 9

Transplantation tolerance: The concept and its applicability

Dong VM, Womer KL, Sayegh MH. Transplantation tolerance: The concept and its applicability.
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Abstract: Recent advances have enabled researchers to induce tolerance in animal transplant models. Although it has been relatively easy to do so in rodents, it has been much more difficult to translate such strategies into primates. Understanding the cellular and molecular mechanisms of the alloimmune response has prompted the development of novel strategies that may obviate the need for immunosuppression in humans. Mechanisms of tolerance and promising new therapies, as well as the inherent difficulties in bringing them into clinical practice, are reviewed.

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With improvements in surgical techniques, infectious disease therapy and overall medical care over the past few decades, manipulation of the immune response remains the major barrier to successful organ transplantation. The introduction of cyclosporin A into clinical use in the early 1980s (1) and the development of newer immunosuppressive drugs (2) have led to a significant reduction in acute rejection rates and improvement in short-term allograft survival. However, achieving long-term graft survival and overcoming chronic rejection remain difficult tasks (3, 4). Moreover, these drugs cause non-specific immunosuppression and result in an increased risk of infection, malignancy and cardiovascular

disease. Therefore, the major goal of transplantation research is the development of strategies to induce donor-specific tolerance.

Tolerance has been defined as a state of specific immunologic unresponsiveness to the antigens of the graft in the absence of maintenance immunosuppression (5). However, it is now clear that active immunoregulatory mechanisms may be important in the development and maintenance of tolerance. Perhaps a more accurate definition of tolerance would be the absence of a destructive immune response against the graft in an immunocompetent host (6). *In vivo* criteria for donor-specific tolerance are the absence of acute rejection with prolongation of graft survival and acceptance of second test grafts from the original donor, while maintaining the ability to reject third party grafts. Unfortunately, investigators have only recently started to examine graft morphology and function in long-term surviving 'tolerant' animals. This is important, because long-term graft survival does not necessarily imply tolerance. In fact, tolerance should not only prevent acute rejection but also the alloantigen-dependent component of chronic allograft dysfunction, the major cause of late graft dysfunction and loss in solid organ transplantation (7).

Abbreviations: Ag, antigen; APC, antigen presenting cell; CD, cluster of differentiation; CTLA, cytotoxic T lymphocyte antigen; FasL, Fas ligand; gp39, glycoprotein 39; ICAM, intercellular adhesion molecule; ICOS, inducible co-stimulator; Ig, immunoglobulin; IL, interleukin; IL-2R, interleukin-2 receptor; IFN, interferon; LFA, leukocyte function associated antigen; MHC, major histocompatibility complex; TCR, T-cell receptor; Th, T helper; TNF, tumor necrosis factor.

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(54) Title: METHODS EMPLOYING AND COMPOSITIONS CONTAINING PLAQUE ASSOCIATED MOLECULES FOR PREVENTION AND TREATMENT OF ATHEROSCLEROSIS

(57) Abstract: Methods and compositions employing plaque associated molecules effective in inducing mucosal tolerance and inhibiting inflammatory processes contributing to atheromatous vascular disease and sequelae are provided.

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METHODS EMPLOYING AND COMPOSITIONS CONTAINING
PLAQUE ASSOCIATED MOLECULES FOR PREVENTION AND
TREATMENT OF ATHEROSCLEROSIS

5 FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to atheromatous plaque associated molecules for prevention and treatment of atherosclerosis and related disease and, more particularly, to methods and compositions employing plaque associated molecules effective in inducing mucosal tolerance and inhibiting
10 inflammatory processes contributing to atheromatous vascular disease and sequelae.

Cardiovascular disease is a major health risk throughout the industrialized world. Atherosclerosis, the most prevalent of cardiovascular diseases, is the principal cause of heart attack, stroke, and gangrene of the
15 extremities, and as such, the principal cause of death in the United States. Atherosclerosis is a complex disease involving many cell types and molecular factors (for a detailed review, see Ross, 1993, Nature 362: 801-809). The process, which occurs in response to insults to the endothelium and smooth muscle cells (SMCs) of the wall of the artery, consists of the formation of
20 fibrofatty and fibrous lesions or plaques, preceded and accompanied by inflammation. The advanced lesions of atherosclerosis may occlude the artery concerned, and result from an excessive inflammatory-fibroproliferative response to numerous different forms of insult. For example, shear stresses are thought to be responsible for the frequent occurrence of atherosclerotic
25 plaques in regions of the circulatory system where turbulent blood flow occurs, such as branch points and irregular structures.

The first observable event in the formation of an atherosclerotic plaque occurs when inflammatory cells such as monocyte-derived macrophages adhere to the vascular endothelial layer and transmigrate through to the sub-
30 endothelial space. Elevated plasma LDL levels lead to lipid engorgement of the vessel walls, with adjacent endothelial cells producing oxidized low

density lipoprotein (LDL). In addition, lipoprotein entrapment by the extracellular matrix leads to progressive oxidation of LDL by lipoxygenases, reactive oxygen species, peroxynitrite and/or myeloperoxidase as well as other oxidizing compounds. These oxidized forms of LDLs are then taken up in
5 large amounts by vascular cells through scavenger receptors expressed on their surfaces.

Lipid-filled monocytes and smooth-muscle derived cells are called foam cells, and are the major constituent of the fatty streak. Interactions between foam cells and the endothelial and smooth muscle cells surrounding
10 them produce a state of chronic local inflammation which can eventually lead to activation of endothelial cells, increased macrophage apoptosis, smooth muscle cell proliferation and migration, and the formation of a fibrous plaque (Hajjar, DP and Haberland, ME, J.Biol Chem 1997 Sep 12; 272(37):22975-78). Such plaques occlude the blood vessels concerned and thus restrict the
15 flow of blood, resulting in ischemia, a condition characterized by a lack of oxygen supply in tissues of organs due to inadequate perfusion. When the involved arteries block the blood flow to the heart, a person is afflicted with a 'heart attack'; when the brain arteries occlude, the person experiences a stroke. When arteries to the limbs narrow, the result is severe pain, decreased physical
20 mobility and possibly the need for amputation.

Oxidized LDL has been implicated in the pathogenesis of atherosclerosis and atherothrombosis, by its action on monocytes and smooth muscle cells, and by inducing endothelial cell apoptosis, impairing anticoagulant balance in the endothelium. Oxidized LDL also inhibits anti-
25 atherogenic HDL-associated breakdown of oxidized phospholipids (Mertens, A and Holvoet, P, FASEB J 2001 Oct; 15(12):2073-84). This association is also supported by many studies demonstrating the presence of oxidized LDL in the plaques in various animal models of atherogenesis; the retardation of atherogenesis through inhibition of oxidation by pharmacological and/or
30 genetic manipulations; and the promising results of some of the

interventional trials with anti-oxidant vitamins (see, for example, Witztum J and Steinberg, D, Trends Cardiovasc Med 2001 Apr-May;11(3-4):93-102 for a review of current literature). Indeed, oxidized LDL and malondialdehyde (MDA)-modified LDL have been recently proposed as accurate blood markers for 1st and 2nd stages of coronary artery disease (US Pat. Nos. 6,309,888 to Holvoet et al and 6,255,070 to Witztum, et al).

Reduction of LDL oxidation and activity has been the target of a number of suggested clinical applications for treatment and prevention of cardiovascular disease. Bucala, et al (US Pat. No. 5869534) discloses methods for the modulation of lipid peroxidation by reducing advanced glycosylation end product, lipid characteristic of age-, disease- and diabetes-related foam cell formation. Tang et al, at Incyte Pharmaceuticals, Inc. (US Pat. No. 5,945,308) have disclosed the identification and proposed clinical application of a Human Oxidized LDL Receptor in the treatment of cardiovascular and autoimmune diseases and cancer.

Another abundant atherogenesis-related plaque component is Beta 2-Glycoprotein I. Beta 2-Glycoprotein I (Beta2GPI) is a 50-kDa molecule that acts as an anticoagulant in *in-vitro* assays. Although the exact role of Beta2GPI in atherogenesis has yet to be elucidated, several relevant properties have been observed: 1) it is able to bind negatively charged phospholipids or phospholipid-expressing cells (apoptotic cells, activated platelets); 2) it is able to bind to modified cellular surfaces, enhancing their clearance by scavenging macrophages (Chonn A, et al J Biol Chem 1995; 270: 25845-49; and Thiagarajan P, et al Arterioscler Thromb Vasc Biol 1999; 19:2807-11); and 3) it is an important target for binding of autoimmune antiphospholipid antibodies (aPLs). Beta2GPI has to undergo structural alteration in order to be recognized by aPLs. This alteration may be initiated, for example, by binding to negatively charged phospholipids or high binding plates, but also *in vivo* by binding apoptotic cells that express phosphatidylserine.

Recent studies investigating the importance of anti Beta2GPI antibodies in promoting a procoagulant state have focused on the effects of these antibodies on cellular and protein components of the coagulation system (endothelial cells, platelets and macrophages; tissue factor and coagulation factors). These studies indicate that anti Beta2GPI antibodies prevent the deactivation of platelets, sustaining their phagocytic clearance; interact with late endosomes of human endothelial cells; and suppress the inhibitory activity of the tissue factor pathway inhibitor. This association with coagulation events is consistent with Beta2GPIs proposed function in the prothrombotic antiphospholipid syndrome (APLS). Both US Pat Nos 5998223 and 5344758 (to Matsuura, et al and Krilis, et al, respectively) disclose the application of anti Beta2GPI antibodies, some to cryptic epitopes, for diagnostics in APLS and SLE. However, no therapeutic applications are disclosed by the authors.

A third important plaque-related component associated with atherogenesis is the 60/65 kDa heat shock protein (HSP). This mitochondrial protein is a member of the HSP family, which constitutes nearly 24 proteins displaying high degree of sequence homologies between different species. These proteins, as their name implies, are expressed in response to stresses including exposure to free radicals, heat, mechanical shear stress, infections and cytokines, and protect against unfolding and denaturation of cellular proteins. This has led to their designation as molecular 'chaperones'. However, HSP function may have undesired consequences, since over expression of HSPs may, under certain conditions promote an autoimmune reaction with resultant tissue damage. The mechanisms responsible for the HSP immune mediated damage are as yet unclear: it is presumed that cryptic, "non-self" neo-epitopes are exposed following their upregulation. Alternatively, it was suggested that cross-reaction exists between self-HSP and 'foreign' HSP epitopes introduced following infections which may trigger a pathological, autoimmune response against native HSP. Support for the involvement of HSP in autoimmunity is provided by studies documenting enhanced autoantibody as

well as cellular response to HSP 60/65 in several autoimmune diseases (Schoenfeld, Y et al Autoimmunity 2000 Sep; 15(2):199-202; US Pat No. 6130059 to Covacci, et al; and Gromadza G, et al Cerebrovascul Dis 2001, Oct; 12(3):235-39).

5 The link between HSP 65 and atherosclerosis was initially recognized by George Wick's group, who found that normocholesterolemic rabbits immunized with different antigens developed atherosclerosis, provided the preparation used for immunization contained complete Freund's adjuvant (CFA)(Xu, Q, et al Arterioscler Thromb 1992;12:789-99). Since the major
10 constituent of CFA is heat killed mycobacterium tuberculosis, the principal component of which is the HSP-65, they reasoned that the immune response towards this component led to the development of atherosclerosis. This was confirmed when these authors demonstrated that immunization of animals with HSP 65 produced pronounced atherosclerosis, and that T cells from
15 experimentally atherosclerotic rabbits overexpressed HSP-65, indicating a localized immune reaction restricted to the stressed arterial vessel. The importance of endogenous HSP-65 in atherogenesis was further demonstrated by the acceleration of fatty streak formation following HSP-65 (or Mycobacterium tuberculosis) immunization of naïve mice (George J, et al
20 Arterioscler Thromb Vasc Biol 1999; 19:505-10;).

 Involvement of humoral immune mechanisms in response to HSP-65 were observed in atherosclerosis: a marked correlation has been found between high levels of anti-HSP65 antibodies and the extent of sonographically estimated carotid narrowing in a screen of healthy individuals
25 (Xu Q. et al Lancet 1993; 341: 255-9; Xu Q. et al Circulation 1999; 100(11):1169-74). In addition, in-vitro experiments with cultured endothelial cells have demonstrated the concentration and time dependent induction of endothelial cell adhesion to monocytes and granulocytes following incubation with HSP65.

The association of HSP 65 with atherogenesis has led to a number of proposed therapeutic applications. Observing that immune reactivity to HSP 65 correlated with both microbial (e.g. *H. pylori*) infection and atherosclerosis, Bernie et al (Eur Heart J 1998; 19:366-7) proposed antibiotic therapy for reduction of infection and anti- HSP antibodies. Similarly, Covacci, et al (US Pat No. 6130059) disclosed the use of *H. pylori* HSPs, and related peptides, for diagnostic and therapeutic applications in atherosclerosis.

Atherosclerosis and autoimmune disease

Because of the presumed role of the excessive inflammatory-fibroproliferative response in atherosclerosis and ischemia, a growing number of researchers have attempted to define an autoimmune component of vascular injury. In autoimmune diseases the immune system recognizes and attacks normally non-antigenic body components (autoantigens), in addition to attacking invading foreign antigens. The autoimmune diseases are classified as auto- (or self-) antibody mediated or cell mediated diseases. Typical autoantibody mediated autoimmune diseases are myasthenia gravis and idiopathic thrombocytopenic purpura (ITP), while typical cell mediated diseases are Hashimoto's thyroiditis and type I (Juvenile) Diabetes.

The recognition that immune mediated processes prevail within atherosclerotic lesions stems from the consistent observation of lymphocytes and macrophages in the earliest stages, namely the fatty streaks. These lymphocytes, which include a predominant population of CD4+ cells (the remainder being CD8+ cells) were found to be more abundant than macrophages in early lesions, as compared with the more advanced lesions, in which this ratio tends to reverse. These findings posed questions as to whether they reflect a primary immune sensitization to a possible antigen or alternatively, result from previously induced local tissue damage. Regardless of the factors responsible for the recruitment of these inflammatory cells to the early plaque, they seem to exhibit an activated state manifested by concomitant expression of MHC class II HLA-DR and interleukin (IL) receptor as well as

leukocyte common antigen (CD45R0) and the very late antigen 1 (VLA-1) integrin. Thus, the inflammatory reaction of the early stages of the atherosclerotic lesion may be either the primary initiating event leading to the production of various cytokines by the local cells (i.e endothelial cells, macrophages, smooth muscle cells and inflammatory cells), or one form of the immune system's response to the hazardous process. Some of the cytokines which have been shown to be upregulated by the resident cells include TNF- α , IL-1, IL-2, IL-6, IL-8, IFN- γ and monocyte chemoattractant peptide-1 (MCP-1). Platelet derived growth factor (PDGF) and insulin-like growth factor (ILGF) which are expressed by all cellular constituents within atherosclerotic plaques have also been shown to be overexpressed, thus possibly intensifying the preexisting inflammatory reaction by a co-stimulatory support in the form of a mitogenic and chemotactic factor. Recently, Uyemura et al. (J Clin Invest 1996; 97: 2130-2138) have elucidated type 1 T-cell cytokine pattern in human atherosclerotic lesions exemplified by a strong expression of IFN- γ but not IL-4 mRNA in comparison with normal arteries. Furthermore, IL-12 - a T-cell growth factor produced primarily by activated monocytes and a selective inducer of Th1 cytokine pattern, was found to be overexpressed within lesions as manifested by the abundance of its major heterodimer form p70 and p40 (its dominant inducible protein) mRNA.

Similar to the strong evidence for the dominance of the cellular immune system within the atherosclerotic plaque, there is also ample data supporting the involvement of the local humoral immune system. Thus, deposition of immunoglobulins and complement components has been shown in the plaques in addition to the enhanced expression of the C3b and C3Bi receptors in resident macrophages.

Valuable clues with regard to the contribution of immune mediated inflammation to the progression of atherosclerosis come from animal models. Immunocompromised mice (class I MHC deficient) tend to develop

accelerated atherosclerosis as compared with immune competent mice. Additionally, treatment of C57BL/6 mice (Emeson EE and Shen ML Am J Pathol 1993; 142: 1906-1915) and New-Zealand White rabbits (Roselaar SE, et al J Clin Invest 1995; 96: 1389-1394) with cyclosporin A, a potent
5 suppressor of IL-2 transcription resulted in a significantly enhanced atherosclerosis under "normal" lipoprotein "burden". These latter studies may provide insight into the possible roles of the immune system in counteracting the self-perpetuating inflammatory process within the atherosclerotic plaque.

Atherosclerosis is not a classical autoimmune disease, although some of
10 its manifestations such as the production of the plaque that obstructs the vasculature may be related to aberrant immune responsiveness. In classical autoimmune disease, one can often define very clearly the sensitizing autoantigen attacked by the immune system and the component(s) of the immune system which recognize the autoantigen (humoral, i.e. autoantibody or
15 cellular, i.e. lymphocytes). Above all, one can show that by passive transfer of these components of the immune system the disease can be induced in healthy animals, or in the case of humans the disease may be transferred from a sick pregnant mother to her offspring. Many of the above are not prevailing in atherosclerosis. In addition, the disease definitely has common risk factors
20 such as hypertension, diabetes, lack of physical activity, smoking and others, the disease affects elderly people and has a different genetic preponderance than in classical autoimmune diseases.

Treatment of inflammatory disease may be directed towards suppression or reversal of general and/or disease-specific immune reactivity.
25 Thus Aiello, for example (US Pat. Nos. 6,034,102 and 6,114,395) discloses the use of estrogen-like compounds for treatment and prevention of atherosclerosis and atherosclerotic lesion progression by inhibition of inflammatory cell recruitment. Similarly, Medford et al (US Pat. No. 5,846,959) disclose methods for the prevention of formation of oxidized PUFA, for treatment of
30 cardiovascular and non-cardiovascular inflammatory diseases mediated by the

cellular adhesion molecule VCAM-1. Furthermore, Falb (US Pat. No. 6,156,500) designates a number of cell signaling and adhesion molecules abundant in atherosclerotic plaque and disease as potential targets of anti-inflammatory therapies.

5 Since oxidized LDL, Beta2GPI and HSP 65 have been clearly implicated in the pathogenesis of atherosclerosis (see above), the contribution of these prominent plaque components to autoimmunity in atheromatous disease processes has been investigated.

Immune responsiveness to plaque associated molecules

10 It is known that Ox LDL is chemotactic for T-cells and monocytes. Ox LDL and its byproducts are also known to induce the expression of factors such as monocyte chemotactic factor 1, secretion of colony stimulating factor and platelet activating properties, all of which are potent growth stimulants. The active involvement of the cellular immune response in atherosclerosis has
15 recently been substantiated (Stemme S, et al, Proc Natl Acad Sci USA 1995; 92: 3893-97), who isolated CD4+ within plaques clones responding to Ox LDL as stimuli. The clones corresponding to Ox LDL (4 out of 27) produced principally interferon- γ rather than IL-4. It remains to be seen whether the above T-cell clones represent mere contact with the cellular immune system
20 with the inciting strong immunogen (Ox LDL) or that this reaction provides means of combating the apparently indolent atherosclerotic process.

The data regarding the involvement of the humoral mechanisms and their meaning are much more controversial. One recent study reported increased levels of antibodies against MDA-LDL, a metabolite of LDL
25 oxidation, in women suffering from heart disease and/or diabetes (Dotevall, et al., Clin Sci 2001 Nov; 101(5): 523-31). Other investigators have demonstrated antibodies recognizing multiple epitopes on the oxidized LDL, representing immune reactivity to the lipid and apolipoprotein components (Steineroova A, et al., Physiol Res 2001;50(2): 131-41) in atherosclerosis and
30 other diseases, such as diabetes, renovascular syndrome, uremia, rheumatic

fever and lupus erythematosus. Several reports have associated increased levels of antibodies to Ox LDL with the progression of atherosclerosis (expressed by the degree of carotid stenosis, severity of peripheral vascular disease etc.). Most recently, Sherer et al (Cardiology 2001;95(1):20-4) demonstrated elevated levels of antibodies to cardiolipin, β -2GPI and oxLDL, but not phosphatidylcholine or endothelial cells in coronary heart disease. Thus, there seems to be a consensus as to the presence of anti-plaque-component antibodies in the form of immune complexes within atherosclerotic plaque.

Antibodies to Ox LDL have been implicated in both normal and pathological lipoprotein metabolism. Thus, it is known that immune complexes of Ox LDL and its corresponding antibodies are taken up more efficiently by macrophages in suspension as compared with Ox LDL. No conclusions can be drawn from this consistent finding on the pathogenesis of atherosclerosis since the question of whether the accelerated uptake of Ox LDL by the macrophages is beneficial or deleterious has not yet been resolved.

Important data as to the significance of the humoral immune system in atherogenesis comes from animal models: hyperimmunization of LDL-receptor deficient rabbits with homologous oxidized LDL, resulted in the production of high levels of anti-Ox LDL antibodies and was associated with a significant reduction in the extent of atherosclerotic lesions. Likewise, a decrease in plaque formation followed the immunization of rabbits with cholesterol rich liposomes and stimulation of production of anti-cholesterol antibodies; however, this effect was accompanied by a undesirable 35% reduction in very low density lipoprotein cholesterol levels.

Regarding the immunogenicity of Beta2GPI, it has been shown that Beta2GPI serves as a target antigen for an immune-mediated attack, influencing the progression of atherosclerosis in humans and mice. George J et al. immunized LDL-receptor deficient mice with Beta2GPI, producing a pronounced humoral immune response to human Beta2GPI, and larger early

atherosclerotic lesions in comparison with controls (George J, et al *Circulation* 1998; 15:1108-15). Afek A, et al obtained similar results in atherosclerosis-prone apolipoprotein-E-knockout mice immunized once with human Beta2GPI and fed a high fat diet for 5 weeks (Afek A et al. *Pathobiology* 1999;67:19-
5 25).

Further, although immune reactivity to Beta2GPI in humans with the prothrombotic antiphospholipid syndrome has traditionally been attributed to the presence of autoantibodies to Beta2GPI, recent observations have indicated the importance of a cellular immune response to Beta2GPI. T-cells reactive
10 with Beta2GPI have been demonstrated in the peripheral blood of patients with antiphospholipid syndrome. These T cells displayed a T-helper-1 phenotype (secreting the proinflammatory (and proatherogenic) cytokine interferon-) and were also capable of inducing tissue factor production (Visvanathan S, and McNiel HP. *J Immunolog* 1999; 162:6919-25). Taken together, the abundant
15 data gathered to date regarding anti Beta2GPI (for review see Roubey RA, *Curr Opin Rheumatol* 2000; 12:374-378), indicates that the immune response to this plaque related antigen may play a significant role in influencing the size and composition of atherosclerotic plaque.

Finally, there exists a significant dependency in the antigenicity, and
20 pathogenicity of oxidized phospholipids and Beta2GPI. As mentioned above, some of the autoimmune epitopes associated with minimally modified LDL and Beta2GPI are cryptic. Kyobashi, et al (*J Lipid Res* 2001; 42:697-709), and Koike, et al (*Ann Med* 2000; 32:Suppl I 27-31) have identified a macrophage-activating oxLDL specific ligand present only with Beta2GPI- OxLDL
25 complex formation. This ligand was recognized by APLS-specific autoantibodies. Thus, both the pathogenic role of oxidized LDL and other plaque components, and their importance as autoantigens in atherosclerosis, as well as other diseases, have been extensively demonstrated in laboratory and clinical studies.

Mucosal Tolerance in Treatment of Autoimmune Disease

Recently, new methods and pharmaceutical formulations have been found that are useful for treating autoimmune diseases (and related T-cell mediated inflammatory disorders such as allograft rejection and retroviral-associated neurological disease). These treatments induce tolerance, orally or mucosally, e.g. by inhalation, using as tolerizers autoantigens, bystander antigens, or disease-suppressive fragments or analogs of autoantigens or bystander antigens. Such treatments are described, for example, in US Pat. No. 5,935,577 to Weiner et al. Autoantigens and bystander antigens are defined below (for a general review of mucosal tolerance see Nagler-Anderson, C., Crit Rev Immunol 2000;20(2):103-20). Intravenous administration of autoantigens (and fragments thereof containing immunodominant epitopic regions of their molecules) has been found to induce immune suppression through a mechanism called clonal anergy. Clonal anergy causes deactivation of only immune attack T-cells specific to a particular antigen, the result being a significant reduction in the immune response to this antigen. Thus, the autoimmune response-promoting T-cells specific to an autoantigen, once anergized, no longer proliferate in response to that antigen. This reduction in proliferation also reduces the immune reactions responsible for autoimmune disease symptoms (such as neural tissue damage that is observed in multiple sclerosis; MS). There is also evidence that oral administration of autoantigens (or immunodominant fragments) in a single dose and in substantially larger amounts than those that trigger "active suppression" may also induce tolerance through anergy (or clonal deletion).

A method of treatment has also been disclosed that proceeds by active suppression. Active suppression functions via a different mechanism from that of clonal anergy. This method, discussed extensively in PCT Application PCT/US93/01705, involves oral or mucosal administration of antigens specific to the tissue under autoimmune attack. These are called "bystander antigens". This treatment causes regulatory (suppressor) T-cells to be induced in the gut-

associated lymphoid tissue (GALT), or bronchial associated lymphoid tissue (BALT), or most generally, mucosa associated lymphoid tissue (MALT) (MALT includes GALT and BALT). These regulatory cells are released in the blood or lymphatic tissue and then migrate to the organ or tissue afflicted by the autoimmune disease and suppress autoimmune attack of the afflicted organ or tissue. The T-cells elicited by the bystander antigen (which recognize at least one antigenic determinant of the bystander antigen used to elicit them) are targeted to the locus of autoimmune attack where they mediate the local release of certain immunomodulatory factors and cytokines, such as transforming growth factor beta (TGF beta), interleukin-4 (IL-4), and/or interleukin-10 (IL-10). Of these, TGF-beta is an antigen-nonspecific immunosuppressive factor in that it suppresses immune attack regardless of the antigen that triggers the attack. (However, because oral or mucosal tolerization with a bystander antigen only causes the release of TGF-beta in the vicinity of autoimmune attack, no systemic immunosuppression ensues.) IL-4 and IL-10 are also antigen-nonspecific immunoregulatory cytokines. IL-4 in particular enhances (T helper 2) Th₂ response, i.e., acts on T-cell precursors and causes them to differentiate preferentially into Th₂ cells at the expense of Th₁ responses. IL-4 also indirectly inhibits Th₁ exacerbation. IL-10 is a direct inhibitor of Th₁ responses. After orally tolerizing mammals afflicted with autoimmune disease conditions with bystander antigens, increased levels of TGF-beta, IL-4 and IL-10 are observed at the locus of autoimmune attack (Chen, Y. et al., Science, 265:1237-1240, 1994). The bystander suppression mechanism has been confirmed by von Herreth et al., (J. Clin. Invest., 96:1324-1331, September 1996).

More recently, oral tolerance has been effectively applied in treatment of animal models of inflammatory bowel disease by feeding probiotic bacteria (Dunne, C, et al., Antonie Van Leeuwenhoek 1999 Jul-Nov;76(1-4):279-92), autoimmune glomerulonephritis by feeding glomerular basement membrane (Reynolds, J. et al., J Am Soc Nephrol 2001 Jan;12(1): 61-70) experimental

allergic encephalomyelitis (EAE, which is the equivalent of multiple sclerosis or MS), by feeding myelin basic protein (MBP), adjuvant arthritis and collagen arthritis, by feeding a subject with collagen and HSP-65, respectively. A Boston based company called Autoimmune has carried out several human experiments for preventing diabetes, multiple sclerosis, rheumatoid arthritis and uveitis. The results of the clinical trials have been less impressive than the animal experiments, however there has been some success with the prevention of arthritis.

Oral tolerance to autoantigens found in atherosclerotic plaque lesions has also been investigated. Study of the epitopes recognized by T-cells and Ig titers in clinical and experimental models of atherosclerosis indicated three candidate antigens for suppression of inflammation in atheromatous lesions: oxidized LDL, the stress-related heat shock protein HSP 65 and the cardiolipin binding protein beta 2GP1. US Patent Application 09/806,400 to Shoenfeld et al (filed Sept 30, 1999), which is incorporated herein in its entirety, discloses the reduction by approximately 30% of atherogenesis in the arteries of genetically susceptible LDL receptor deficient mice (LDL-RD) fed oxidized human LDL. Although significant inhibition of atherogenesis was achieved, presumably via oral tolerance, no identification of specific lipid antigens or immunogenic LDL components was made. Another obstacle encountered was the inherent instability of the orally fed antigen in vivo, due to digestive breakdown, and uptake of oxidized LDL by the liver and cellular immune mechanisms. It is plausible that a mucosal route of administration other than feeding (oral) would have provided tolerance of greater efficiency.

The induction of immune tolerance and subsequent prevention or inhibition of autoimmune inflammatory processes has been demonstrated using exposure to suppressive antigens via mucosal sites other than the gut. The membranous tissue around the eyes, the middle ear, the respiratory and other mucosa, and especially the mucosa of the nasal cavity, like the gut, are exposed to many invading as well as self- antigens and possess mechanisms

for immune reactivity. Thus, Rossi, et al (Scand J Immunol 1999 Aug;50(2):177-82) found that nasal administration of gliadin was as effective as intravenous administration in downregulating the immune response to the antigen in a mouse model of celiac disease. Similarly, nasal exposure to acetylcholine receptor antigen was more effective than oral exposure in delaying and reducing muscle weakness and specific lymphocyte proliferation in a mouse model of myasthenia gravis (Shi, FD. et al, J Immunol 1999 May 15; 162 (10): 5757-63). Therefore, immunogenic compounds intended for mucosal as well as intravenous or intraperitoneal administration should be adaptable to nasal and other membranous routes of administration.

Thus, there is clearly a need for novel methods of employing, and compositions of plaque associated molecules capable of superior tolerizing immunogenicity in mucosal, especially nasal, administration.

15 SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a pharmaceutical composition for prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, comprising, as an active ingredient, a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule, or pharmaceutical salts thereof, the composition further comprising a pharmaceutically acceptable carrier, the pharmaceutical composition being designed for mucosal administration.

According to an additional aspect of the present invention there is provided an assay for determining the effect of mucosal administration of plaque components on atherosclerosis-related disease or condition, the assay effected by mucosally administering to a subject having an atherosclerosis-related disease or condition at least an antigenic portion of at least one plaque associated molecule and assessing at least one indicator of atherogenesis in the

subject to thereby determine the effect of mucosal administration of the at least an antigenic portion of the at least one plaque associated molecule on the atherosclerosis-related disease or condition.

According to yet another aspect of the present invention there is provided a method of prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, the method comprising mucosally administering a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule to the subject.

According to further features in preferred embodiments of the invention described below, the plaque associated molecule is selected from the group consisting of oxidized LDL, Beta2GPI, HSP and derivatives thereof.

According to still further features in preferred embodiments of the invention described below, the antigenic portion of at least one plaque associated molecule is a naturally occurring molecule or a synthetic molecule.

According to further features in preferred embodiments of the invention described below, the pharmaceutical composition is designed for nasal, respiratory, otic and/or conjunctival administration.

According to yet further features in preferred embodiments of the invention described below the at least an antigenic portion of the at least one plaque associated molecule is selected so as to reduce immune reactivity to plaque components in said subject.

According to further features in preferred embodiments of the invention described below, the pharmaceutical composition is packaged and identified for use in the prevention and/or treatment of at least one disorder selected from the group consisting of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis.

According to further features in preferred embodiments of the invention described below the pharmaceutical composition further comprises a therapeutically effective amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a novel method of inducing superior immune tolerance by mucosal administration of plaque associated molecules, thereby inhibiting atherosclerosis and other plaque related diseases.

5

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred
10 embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more
15 detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 illustrates inhibition of early atherogenesis in apo-E deficient
20 mice by nasal tolerance induced by administration of low doses of plaque associated molecules. 9-13 week old apo-E deficient mice were exposed intranasally, with mild sedation, to 3 doses of 10 µg/mouse each HSP 65 (HSP-65)(n=12), human oxidized LDL (H-oxLDL)(n=14), human Beta2GPI (B2gpi)(n=13), bovine serum albumin (BSA) or sham exposure to saline

(PBS)(n=12). All mice received the atherogenic "Western" diet following last exposure. Atherogenesis is expressed as the area of atheromatous lesions in the aortic sinus 5 weeks following the 3rd exposure.

FIG. 2 illustrates superior inhibition of early atherogenesis in apo-E deficient mice by mucosal tolerance induced by intranasal exposure to exceedingly low doses of HSP 65. Nasal tolerance was induced in 12-16 week old apo-E deficient mice by intranasal administration of 3 doses of 1µg/mouse HSP65 (HSP-65 low)(n=16) or 10µg/mouse HSP65 (HSP-65 high)(n=14) every other day for 5 days. Control mice were exposed intranasally to an identical volume (10µl) of bovine serum albumin, 10µg/mouse (BSA)(n=14), or sham exposure to PBS (PBS)(n=14). All mice received the atherogenic "Western" diet following last exposure. Atherogenesis is expressed as the area of atheromatous lesions in the aortic sinus 5 weeks after the last nasal exposure.

FIG. 3 illustrates superior suppression of immune reactivity to atherosclerotic plaque antigens induced by nasal exposure to human Beta2GPI. 5 week old male apo-E deficient mice were exposed intranasally to 10µg/mouse human Beta2GPI (H-b2-nt)(n=3); or alternately fed, by gavage, with 100µg/mouse human Beta2GPI (H-b2-ot)(n=3) in 0.2 ml PBS; or fed PBS alone (PBS)(n=3) every other day for 5 days. One week following the last feeding the mice were sensitized with a single subcutaneous injection of 10µg/mouse human Beta2GPI in 0.1 ml volume. Ten days later T-cells from inguinal lymph node were prepared as described in Materials and Methods section that follows, and exposed to the sensitizing human Beta2GPI antigen for in-vitro assessment of proliferation. Proliferation, indicating immune reactivity, is expressed as the ratio between incorporation of labeled thymidine into the T-cell's DNA in the presence and absence of human Beta2GPI antigen (stimulation index, S.I.).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of methods and compositions employing plaque associated molecules effective in inducing mucosal tolerance to atheroma related antigens, thus inhibiting inflammatory processes contributing
5 to atheromatous vascular disease and sequelae.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the
10 details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

15 Experimental and clinical evidence indicates a causative role for plaque associated antigens in the etiology of the excessive inflammatory response in atherosclerosis. Both cellular and humoral immune reactivity to the plaque associated molecules oxidized LDL, Beta2GPI and HSP 65 have been demonstrated, suggesting an important anti-oxidized LDL auto-immune
20 component in atherogenesis. Thus, oxidized LDL, Beta2GPI and HSP 65, and components thereof, have been the targets of numerous therapies for prevention and treatment of heart disease, cerebral-vascular disease and peripheral vascular disease.

Prior art teaches the application of plaque associated antigens for
25 detection and diagnosis of atherosclerosis and other plaque- and thrombosis related conditions. For example, Holvoet (US Pat No. 6,309,888) teaches the use of stage specific plaque associated antigens oxLDL and MDA-LDL for screening for Coronary Artery disease. Similarly, others (US Pat Nos. 5,998,223 and 5,344,758 to Matsuura, et al and Krilis, et al, respectively) have
30 disclosed the use of anti beta-2GPI antibodies to screen for serum indicators of

APLS, SLE and atherosclerosis. The abovementioned disclosures propose diagnostic applications alone, and fail to recognize the therapeutic potential of these plaque associated molecules.

Although the role of immune response in the etiology and progression
5 of atherosclerosis and other plaque related diseases remains controversial (see Meir, K, et al, International Atherosclerosis Soc. 2001 Commentary), many immune-based therapies have been proposed for atherosclerosis. General methods of reducing immune response in inflammatory and hyperreactive conditions are taught in, for example US Patent Nos 6,277,969; 5,698,195 and
10 5,656,272 to Le et al, and 6,224,902 to Alving, et al, International Patent Application Nos. 001 001 2514 to Shurkovitz et al and 20010051156 A1 to Zeng. However, the proposed reduction or removal of mediators of immune reactivity, such as cytokines, tumor necrosis factor (TNF) and other pathogenic factors requires ongoing costly and potentially dangerous methods such as
15 immunoadsorption of blood and prolonged anti-cytokine administration. Furthermore, no application to treatment of atherosclerosis or plaque-related disease is disclosed.

Specific immunotherapy with plaque associated antigens has also been proposed. Bumol, et al, Calenoff, et al and Takano, et al (US Pat Nos.
20 5,196,324; 6,025,477 and 5,110,738, respectively) disclose the use of crude, poorly defined fractionated plaque preparations for immunization, monoclonal Ab preparation, diagnosis and treatment of atherosclerosis. These antigens, protein and lipid fractions of atheromatous tissue, are poorly defined, impractical for therapeutic use, and potentially hazardous in prolonged
25 treatment.

Prior art teaches immunotherapy directed against oxidized LDL for treatment and prevention of atherosclerosis. US Pat No. 6,225,070 to Witztum, et al discloses the use of mAb to oxidized LDL for inhibition of oxidized LDL binding to macrophages and foam cell formation. Similarly,
30 McGregor, et al (International Patent Application EP1162458 A1) disclose

methods for specific modulation of oxidized LDL uptake and transport by macrophages. US Pat Nos 5,733,524 and 5,733,933 to Bucala, et al disclose the reduction of specific anti-oxidized LDL immune response by reduction of Advanced Glycosylation End product lipids (AGE-lipids). None of the proposed therapies teach active immunization against oxidized LDL, and require prolonged therapy regimens.

Zhou, et al (Arterioscler Thromb Vasc Biol, 2001;21:108) achieved a significant reduction in early plaque formation in mice following footpad immunization with homogenized plaque or homologous MDA LDL. Palinski et al (PNAS USA 1995;92:821-25) produced similar levels of protection in rabbits immunized with oxidized LDL. However, application of conventional immunization techniques to oxidized LDL is problematic, since the adjuvant preparations required for immunization and boosters have produced accelerated plaque formation in similar regimen of immunization. Furthermore, relatively high doses (100 µgram/ mouse/ injection) of plaque antigen were required for immunity. Mucosal administration and induction of tolerance were not mentioned.

Immune therapy with other plaque antigens has also been proposed. Recent animal and in-vitro studies with Beta2GPI (see George J, et al Rheum Dis Clin North Am 2001;27:603-10; Brey, et al Stroke 2001;32:1701-06; Kyobashi, et al J Lipid Res 2001;42:697-709; Koike T, et al Ann Med 2000;32,Suppl. I:27-31 and Cabral AR et al Am J Med 1996;101:472-81) have demonstrated the association of Beta2GPI with stroke, APLS, atherosclerosis and myocardial infarction. Although cryptic epitopes of the protein were clearly implicated in humoral and cellular immune response to oxidized LDL, none of the abovementioned studies demonstrated protective immunity with the protein. Similarly, studies with HSP 65 (Birnie DH Eur Heart J 1998;19:366-67; Xu Q, et al Circulation 1999;100:1169-74; and Gromadzka G, et al Cerebrovasc Dis 2001;12:235-39) have implicated this plaque

associated antigen in stroke and heart disease, suggesting that humoral immunity may be a triggering factor.

The complexity of plaque antigen immunity in atherosclerosis was demonstrated by Schoenfeld Y, et al (Autoimmunity 2000;15:199-202) who immunized LDL-receptor deficient (KO) mice with both HSP 65 and Beta2GPI protein antigens, producing strong cellular and humoral responses, and enhanced plaque formation. Similar increased atherogenesis was observed with passive transfer of plaque antigen activated lymphocytes. None of the above mentioned studies demonstrated inhibition of atherogenic processes by immune tolerance.

Suppression of immune response to autoantigens in atherosclerosis and related disease has been recently investigated. Victoria et al (US Pat. No. 6,207,160 and 5,844,409) discloses specific non-immunogenic Beta2GPI peptides lacking T cell epitopes for reducing antibody binding of immune cells and inducing B-cell tolerance in APLS, SLE and other diseases. However, no actual protection was demonstrated, and the disclosures emphasize the diagnostic use of the non immunogenic peptides. George J, et al (Atherosclerosis 1998;138:147-52) demonstrated the feasibility of immune suppression by hyperimmunization with MDA LDL and reduction of atherogenesis in mice. However, impractically large doses of antigen were required, and the paradoxical response to immunization with plaque antigens obviates the clinical efficacy of such therapy. Furthermore, none of the abovementioned studies disclose induction of mucosal tolerance for treatment of atherosclerosis.

Oral and mucosal tolerance for suppression and prevention of inflammatory conditions is well known in the art. Examples of candidate conditions, antigens and modes of therapy, can be found, for example in US Pat Nos. 5,935,577; 5,397,771; 4,690,683 to Weiner et al., and International Pat Nos. EP 0886471 A1 and WO 01821951 to Haas, et al. US Pat Application No 09/806,400 to Shoenfeld et al filed Sept 30, 1999, which is

incorporated herein in its entirety, teaches the oral administration of plaque associated antigens for the induction of tolerance in LDL receptor deficient mice. Measuring arterial fatty streak lesion density, the inventors demonstrated that oral administration of oxidized LDL, Beta2GPI and HSP 65 derived from animal sources were each able to produce approximately 30% reduction in atherogenesis. Typically, however, oral administration of antigens presents numerous obstacles to achieving tolerance and accurate dosing: the antigens are acted upon by digestion, altering both concentrations and molecular structure prior to their presentation to the lymphatic tissue of the Peyer's patches. Furthermore, the authors failed to investigate the efficacy of other routes of administration for induction of tolerance, such as mucosal and nasal tolerance. As the above mentioned disclosures clearly demonstrate, the parameters for induction of oral and mucosal tolerance cannot be deduced from antigenic activity in conventional immunization, or even in-vitro results, and must result from extensive empirical experimentation. Indeed, many studies have demonstrated the complexities inherent in manipulating the "balance between reacting and nonreacting" in the immune system. Zivny, et al (Clin Immunol 2001;101:150-68) clearly state that "In general, the response to one (tolerance inducing) antigen could not necessarily predict the response to another". Likewise, Hannihen et al (Diabetes 2001;50:771-75) observed that oral, nasal and respiratory administration of antigens caused appearance of disease symptoms (diabetes), rather than inducing tolerance. Similar inconsistencies in mucosal tolerance have been reported by Fujihashi et al (Acta Odontol Scand 2001;59:301-08), Jiang HK et al (Br J Ophthalmol 2001;85:739-44). Problems in mucosal vaccination strategies have been recently reviewed (Ogra PL, et al, Clin Microbiol Rev 2001;14:430-45; Chen H et al, J Control Release 2000;67:117-28; and Lehner T et al, J Infect Dis 1999;179 Suppl 3:S489-92).

While reducing the present invention to practice, the present inventors have uncovered that nasal administration of plaque associated molecules

results in the induction of mucosal tolerance, suppression of anti-plaque related antigen immune reactivity and protection from atherosclerosis. Mucosal tolerance is advantageous for its greater ease of application, accuracy of dosage and greatly reduced incidence of alteration of the tolerizing molecule by digestive and metabolic processes (especially in non-oral routes of administration). These advantages provide superior protection from atherogenic processes, improved patient compliance and reduced cost of therapy.

Thus, according to one aspect of the present invention there is provided a method of inducing immune tolerance to plaque associated molecules in a subject such as a human being.

The method, according to this aspect of the present invention is effected by administering to a subject (e.g., a human) a therapeutically effective amount of an antigenic portion of at least one plaque associated molecule.

As used herein, the phrase "mucosal administration" is defined as application of any and all compounds and/or compositions to mucosal membranes having component or components of the mucosal associated lymphatic tissue. Non-limiting examples of mucosal administration are buccal, intranasal, otic (middle ear), conjunctival, vaginal, rectal, etc. Mucosal administration excludes, for example, intravenous, subcutaneous and epidural administration.

As used herein, the phrase "plaque associated molecules" is defined as any and all protein, carbohydrate, lipid and nucleic acid molecules, portions thereof (antigenic portions), their derivatives, or combinations thereof physically or functionally related to the etiology, pathogenesis, symptomatology and/or treatment of a plaque related condition or disease. Such molecules may be, for example, plaque components such as oxidized LDL, foam cell components, etc, but may also include humoral and cellular entities, such as antibodies, cytokines, growth factors and T cell receptors.

As used herein, the phrase "antigenic portion" refers to a portion of a molecule capable of eliciting an immune response. For example, in cases where the molecule is a protein (e.g., HSP 65, Beta2GPI) such a portion can include a stretch of 6-8 amino acids that constitute an antigenic epitope.

5 Methods for predicting antigenic portions are well known in the art, for example, DNASTAR'S PROTEAN sequence analysis and prediction module (DNASar, Madison, WI). As such determining antigenic portions of plaque associated molecules suitable for use with the present invention is well within the capabilities of an ordinarily skilled artisan.

10 Plaque associated molecules (as well as fragments, analogs, portions and derivatives thereof) can be purified from natural sources (the tissue or organ where they normally occur) and can also be obtained using recombinant DNA technology, in bacterial, yeast, insect (e.g. baculovirus) and mammalian cells using techniques well-known to those of ordinary skill in the art. Amino
15 acid sequences for many potential and actual plaque associated molecules are known, for example: human Beta2GPI (Accession No AAB21330 to Matsuura, et al), HSP65 (Accession No. AF65546 to Oliviera, et al) and human macrophage LDL scavenger receptor (Accession No. XP_008489 to NCBI Annotation Project).

20 Immune tolerance established using the present methodology can be used in the prevention and/or treatment of disorders associated with plaque formation, including but not limited to atherosclerosis, atherosclerotic cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and in-stent-stenosis. Some non-limiting examples of
25 atherosclerotic cardiovascular disease are myocardial infarction, coronary arterial disease, acute coronary syndromes, congestive heart failure, angina pectoris and myocardial ischemia. Some non-limiting examples of peripheral vascular disease are gangrene, diabetic vasculopathy, ischemic bowel disease, thrombosis, diabetic retinopathy and diabetic nephropathy. Non-limiting
30 examples of cerebrovascular disease are stroke, cerebrovascular inflammation,

cerebral hemorrhage and vertebral arterial insufficiency. Stenosis is occlusive disease of the vasculature, commonly caused by atheromatous plaque and enhanced platelet activity, most critically affecting the coronary vasculature. Restenosis is the progressive re-occlusion often following reduction of occlusions in stenotic vasculature. In cases where patency of the vasculature requires the mechanical support of a stent, in-stent-stenosis may occur, re-occluding the treated vessel.

Several plaque associated molecules are suitable for use with the present method. Examples include, but are not limited to, modified lipids, phospholipids and lipoproteins, apolipoprotein-lipid complexes such as LDL-cardiolipin, specific epitopes of proteinaceous molecules such as HSP and Beta2GPI, foam cell surface antigens such as LDL receptor and smooth muscle components such as troponin.

According to a preferred embodiment of the present invention the plaque associated molecule(s) utilized by the method of the present invention is oxidized LDL, Beta2GPI, HSP 65 and/or derivatives thereof.

According to another preferred embodiment of the present invention, a combination of at least two of the abovementioned molecules is administered to the subject.

The method of the invention may be used for prevention and/or treatment of non-atherosclerosis related diseases. For example, phospholipids, phospholipid metabolites and HSP 65 have been clearly implicated in the pathogenesis, and therefore potential treatment of additional, non-atherosclerosis-related diseases. Such diseases and syndromes include oxidative stress of aging (Onorato JM, et al, Annal N Y Acad Sci 1998 Nov 20;854:277-90), rheumatoid arthritis (RA)(Paimela L, et al. Ann Rheum Dis 1996 Aug;55(8):558-9), juvenile rheumatoid arthritis (Savolainen A, et al, 1995;24(4):209-11), inflammatory bowel disease (IBD)(Sawai T, et al, Pediatr Surg Int 2001 May;17(4):269-74), renal cancer (Noguchi S, et al, Biochem Biophys Res Commun 1992 Jan 31;182(2):544-50), venous and arterial

thromboses (Cabral AR, et al Am J Med 1996;101:472-81), Anti Phospholipid Syndrome (APLS or APS) (Koike T, et al Ann Med 2000;32 Suppl I:27-31), Systemic Lupus Erythematosus (US Pat Nos. 5,344,758 and 6,207,160, to Krilis, et al and Victoria, et al, respectively). Thus, the method of the invention may be used for prevention and/or treatment of non-atherosclerosis related diseases such as aging, RA, juvenile RA, IBD, SLE, APLS, thrombosis and cancer.

The immune tolerance inducing molecules or molecule combinations described hereinabove can be administered *per se*, or in a pharmaceutical composition where they are mixed with suitable carriers or excipients.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of the pharmaceutical composition of the present invention is to facilitate mucosal administration of the immune tolerance inducing molecules to an organism.

Herein the term "active ingredient" refers to the at least an antigenic portion of the plaque associated molecules (e.g. oxidized LDL, HSP 65, and beta2GP-I) or combinations thereof which are accountable for the biological effect (immune tolerance).

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of mucosal administration may, for example, include
5 rectal, buccal, vaginal and especially transnasal, otic, conjunctival and respiratory (including intratracheal) application.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating,
10 emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which,
15 can be used pharmaceutically. Proper formulation is dependent upon the mucosal route of administration chosen.

The active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer.
20 Penetrants appropriate to the barrier to be permeated may be used in the formulation. Such penetrants are generally known in the art.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use
25 of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a
30 suitable powder base such as lactose or starch.

The pharmaceutical composition of the present invention may be administered to the membranes of the eye, such as the conjunctiva. As such, the composition may be formulated in a liquid or semi-liquid composition, as described above, for application using for example, a drop applicator. Sterility
5 may be ensured by sterilization methods known to one skilled in the art.

The pharmaceutical composition of the present invention may be formulated in rectal and vaginal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

10 The pharmaceutical composition of the present invention may be administered by inhalation. Examples of formulations for tolerizing agents that are administered by inhalation are provided in PCT/US90/07455, filed Dec. 17, 1990. The pharmaceutical formulations for administration by inhalation of the present invention may include, as optional ingredients,
15 pharmaceutically acceptable carriers, diluents, solubilizing and emulsifying agents, and salts of the type that are well-known in the art. Examples of such substances include normal saline solutions, such as physiologically buffered saline solutions, and water.

The route of administration of tolerizing antigens according to this
20 alternate embodiment of the present invention is in an aerosol or inhaled form. The antigens can be administered as dry powder particles or as an atomized aqueous solution suspended in a carrier gas (e.g. air or N₂). Preferred aerosol
pharmaceutical formulations may comprise for example, a physiologically-acceptable buffered saline solution containing between about 1 mg and about
25 300 mg of the antigens.

Dry aerosol in the form of finely divided solid particles of tolerizing antigens that are not dissolved or suspended in a liquid are also useful in the practice of the present invention. The tolerizing antigens may be in the form of dusting powders and comprise finely divided particles having an average
30 particle size of between about 1 and 5 microns, preferably between 2 and 3

microns. Finely divided particles may be prepared by pulverization and screen filtration using techniques well known in the art. The particles may be administered by inhaling a predetermined quantity of the finely divided material, which can be in the form of a powder.

5 Specific non-limiting examples of the carriers and/or diluents that are useful in the by-inhalation pharmaceutical formulations include water and physiologically-acceptable buffered saline solutions such as phosphate buffered saline solutions pH 7.0-8.0. Additional non-limiting examples of suitable carriers or diluents for use in by-inhalation pharmaceutical
10 formulations or dosage forms of the present invention are disclosed in U.S. Pat. No. 5,935,577 to Weiner, et al.

 The pharmaceutical formulations of the present invention may be administered in the form of an aerosol spray using for example, a nebulizer such as those described by Weiner, et al (US Pat No. 5,935,577). The aerosol
15 material is inhaled by the subject to be treated.

 Other systems of aerosol delivery, such as the pressurized metered dose inhaler (MDI) and the dry powder inhaler as disclosed in Newman, S. P. in Aerosols and the Lung, Clarke, S. W. and Davia, D. eds. pp. 197-224, Butterworths, London, England, 1984, can be used when practicing the present
20 invention. Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, Mass.), Schering Corp. (Kenilworth, N.J.) and American Pharmoseal Co. (Valencia, Calif.).

 Pharmaceutical compositions suitable for use in context of the present
25 invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients effective to induce an immune response thus preventing, alleviating or ameliorating symptoms of a disorder (e.g., atherosclerosis).

Ascertaining the optimum regimen for administering the active ingredient(s) is determined in light of the information disclosed herein and well known information concerning administration of mucosally active antigens, and autoantigens. Routine variation of dosages, combinations, and duration of treatment is performed under circumstances wherein the severity of atheromatous development can be measured. Useful dosage and administration parameters are those that result in reduction in inflammatory reaction, including a decrease in number of autoreactive T-cells, or in the occurrence or severity of at least one clinical or histological symptom of the disease.

The pharmaceutical composition of the present invention can be formulated comprising a therapeutically effective amount of additional compound or compounds useful in treating or preventing plaque related disease. In one preferred embodiment the additional compounds are HMGCoA reductase inhibitors (statins), mucosal adjuvants (see, for example, US Pat No. 6,270,758 to Staats, et al), corticosteroids, anti-inflammatory compounds (see, for example US Pat No. 6,297,260 to Bandarage, et al), analgesics, growth factors, toxins and additional tolerizing agents. In addition, it will be appreciated that use of the methods and compositions of the present invention does not preclude the initiation or continuation of other therapies for the abovementioned diseases or conditions, except where of specifically counterindicated.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

In further preferred embodiments of the present invention, cytokine and non-cytokine synergists can be conjoined in the treatment to enhance the effectiveness of mucosal tolerization with plaque associated molecules. Oral and parenteral use of other cytokine synergists (Type I interferons) has been

described in PCT/US95/04120, filed Apr. 07, 1995. Administration of Th2 enhancing cytokines is described in PCT application no. PCT/US95/04512, filed Apr. 07, 1995. For example, IL-4 and IL-10 can be administered in the manner described in PCT/US95/04512.

5 Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage
10 form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

 Dosage amount and interval may be adjusted individually to provide
15 mucosal levels of the active ingredient that are sufficient to induce tolerance. The "tolerizing dosage" will vary for each preparation, but can be estimated from *in vitro* data. Dosages necessary to achieve tolerizing dosage will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

20 Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

 The amount of a composition to be administered will, of course, be
25 dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

 Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack
30 may, for example, comprise an inhaler. The pack or inhaler may be

accompanied by instructions for administration. The pack or inhaler may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

It will be appreciated that administration of the tolerizing compositions and methods of the present invention may be effected by additional non mucosal methods such as intradermal, subcutaneous and intraperitoneal application.

According to another aspect of the present invention, there is provided an assay for determining the effects of mucosal administration of plaque associated molecules on arteriosclerosis related disease or condition, the assay effected by mucosally administering the plaque associated molecule or composition thereof to a subject having such a disease or condition, and assessing at least one indicator of atherogenesis or inflammation. In a preferred embodiment, the plaque associated molecule is oxidized LDL, beta-2-GPI, HSP and/or derivatives thereof. In another embodiment at least an antigenic portion of at least one plaque associated molecule is administered mucosally, the plaque associated molecule being a naturally occurring or synthetic molecule.

Indicators of atherogenesis or inflammation that can be assessed in the context of the assay of the present invention are known to the art. Some non-limiting examples are histological methods such as fatty streak lesion count, and immunological methods such as Stimulation Index, as described herein in

Examples section that follows. Progression of atherosclerosis can be assessed, for example, in atherosclerosis-prone mice maintained on an atherogenic diet (see, for example, George J, et al Circulation 1999;99:2227-30). Inflammation can be assessed by cytological, immunological, biochemical, molecular and genetic techniques known in the art.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include biochemical and immunological techniques. Such techniques are thoroughly explained in the literature. See, for example, "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; and "Methods in Enzymology" Vol. 1-317, Academic Press; Marshak et al., all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The

procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

5

Materials and Experimental Methods

Animals: Apo-E deficient mice used in these experiments are from the atherosclerosis prone strain C57BL/6J-Apo-E^{tm1unc}. Mice homozygous for the Apo-E^{tm1unc} mutations show a marked increase in total plasma cholesterol levels which is unaffected by age or sex. Fatty streaks in the proximal aorta are found at 3 months of age. The lesions increase with age and progress to lesions with less lipid but more elongated cells, typical of a more advanced stage of pre-atherosclerotic lesion.

Strain Development: The Apo-E^{tm1unc} mutant strain was developed in the laboratory of Dr. Nobuyo Maeda at University of North Carolina at Chapel Hill. The 129-derived E14Tg2a ES cell line was used. The plasmid used is designated as pNMC109 and the founder line is T-89. The C57BL/6J strain was produced by backcrossing the Apo-E^{tm1unc} mutation 10 times to C57BL/6J mice (11,12).

The mice were maintained at the Sheba Hospital Animal Facility (Tel-Hashomer, Israel) on a 12-hour light/dark cycle, at 22-24°C and fed a normal fat diet of laboratory chow (Purina Rodent Laboratory Chow No. 5001) containing 0.027% cholesterol, approximately 4.5% total fat, and water, ad libitum. "Western diet" (TD 96125, Harlan Teklad, 42% calories from fat, 43% from carbohydrates and 15% from protein) describes a standardized, high fat atherogenic diet.

Nasal Tolerance: Nasal tolerance was induced by intranasal administration of oxidized LDL, Beta2GPI or HSP65, in a total volume of 10 µl PBS. Intranasal administration was performed on mildly sedated mice (12-16 weeks old), each mouse receiving 3 doses of antigen per dose, in the indicated concentrations, every other day. Atherogenesis was induced by 5

weeks of a Western diet, initiated on the day following the last intranasal administration. Controls received equal amounts of BSA and/or PBS, as indicated, in an identical regimen. Plasma samples were obtained for assessment of cholesterol and triglyceride levels from all mice, and the mice
5 were sacrificed for evaluation of atherosclerosis, as described hereinbelow, after 5 weeks Western diet.

Oral Tolerance: For comparison, oral tolerance to plaque associated molecules was induced by feeding 3 doses of antigen every other day (for a detailed account of induction of oral tolerance, see US Pat Application No
10 09/806,400 to Shoenfeld et al filed Sept 30, 1999), in a similar regimen to the nasal tolerance.

Antigen Preparation

Beta2GPI: Human Beta2GPI was purified from the serum of a healthy adult as described by Gharavi, et al (J Clin Invest 1992;92:1105-09).

15 **Oxidized LDL:** Human LDL (density=1.019- 1.063g/l) was prepared from Plasma of fasting individuals by preparative ultracentrifugation (50,000 rpm/min, 22 min), washing, dialysis against 150mM EDTA, pH 7.4, filtration (0.22 μ m pore size) to remove aggregation, and storage under nitrogen. LDL oxidation was performed by incubation of dialyzed, EDTA-free LDL with
20 copper sulfate (10 μ M) for 24 hours at 37° C. Lipoprotein oxidation was confirmed by analysis of thiobarbituric acid-reactive substances (TBARS), which measures malondialdehyde (MDA) equivalents.

HSP65: Recombinant mycobacterial HSP-65, prepared as described (Prohaszka Z et al, Int Immunol 1999;11:1363-70) was kindly provided by Dr.
25 M. Singh, Braunschweig, Germany.

Immunization: Subcutaneous immunization with human Beta2GPI: Human Beta2GPI was prepared from human plasma pool as described above. For immunization, human Beta2GPI was dissolved in PBS and mixed with equal volumes of Freund's incomplete adjuvant.
30 Immunizations were performed by single subcutaneous injection of 10 μ g

antigen/mouse in 0.1ml volume. Three days following the last mucosal administration of plaque associated molecules the mice received one immunization, and were sacrificed 10 days post immunization.

Cholesterol Level Determination: At the completion of the experiment, 1-1.5 ml of blood was obtained by cardiac puncture into vials containing EDTA, centrifuged to separate plasma. Total plasma cholesterol levels were determined using an automated enzymatic technique (Boehringer Mannheim, Germany).

FPLC Analysis: Fast Protein Liquid Chromatography analysis of cholesterol and lipid content of lipoproteins was performed using Superose 6 HR 10/30 column (Amersham Pharmacia Biotech, Inc, Peapack, NJ) on a FPLC system (Pharmacia LKB. FRAC-200, Pharmacia, Peapack, NJ). A minimum sample volume of 300 μ l (blood pooled from 3 mice was diluted 1:2 and filtered before loading) was required in the sampling vial for the automatic sampler to completely fill the 200 μ l sample loop. Fractions 10-40 were collected, each fraction contained 0.5 ml. A 250 μ l sample from each fraction was mixed with freshly prepared cholesterol reagent or triglyceride reagent respectively, incubated for 5 minutes at 37°C and assayed spectrophotometrically at 500nm.

Assessment of Atherosclerosis: Quantification of atherosclerotic fatty streak lesions was done by calculating the lesion size in the aortic sinus as previously described (George J et al Circulation 1999;99:2227-30) and by calculating the lesion size in the aorta. Briefly, after perfusion with saline Tris EDTA, the heart and the aorta were removed from the animals and the peripheral fat cleaned carefully. The upper section of the heart was embedded in OCT medium (10.24% w/w polyvinyl alcohol; 4.26% w/w polyethylene glycol; 85.50% w/w nonreactive ingredients) and frozen. Every other section (10 μ m thick) throughout the aortic sinus (400 μ m) was taken for analysis. The distal portion of the aortic sinus was recognized by the three valve cusps that

are the junctions of the aorta to the heart. Sections were evaluated for fatty streak lesions after staining with oil-red O. Lesion areas per section were scored on a grid by an observer counting unidentified, numbered specimens. The aorta was dissected from the heart and surrounding adventitious tissue was removed. Fixation of the aorta and Sudan staining of the vessels were performed as previously described (George J et al Circulation 1999;99:2227-30).

Proliferation assays: Mice were exposed to the tested antigen as described for assessment of atherosclerosis, and then immunized (one to three days following the last exposure) subcutaneously with 10 µg Beta2GPI in 0.1 ml PBS, prepared from purified human Beta2GPI as described above.

Proliferation was assayed ten days after immunization with the Beta2GPI as follows: Draining inguinal lymph nodes were prepared by meshing the tissues on 100 mesh screens. Red blood cells were lysed with cold sterile double distilled water (6ml) for 30 seconds and 2ml of NaCl 3.5% was added. Incomplete medium was added (10ml), cells were centrifuged for 7 min at 1,700 rpm, resuspended in RPMI medium and counted in a haemocytometer at 1:20 dilution (10µl cells + 190µl Trypan Blue). Proliferation was measured by the incorporation of [³H] Thymidine into DNA in triplicate samples of 100µl of the packed cells (1×10^6 cells/ml) in a 96 well microtiter plate. Triplicate samples of Beta2GPI (10µg/ml, 100µl/well) were added, cells incubated for 72 hours (37°C, 5% CO₂ and ~98% humidity) and 10µl [³H] Thymidine (0.5µCi/well) was added. After an additional day of incubation the cells were harvested and transferred to glass fiber filters using a cell harvester (Brandel) and counted using β-counter (Lumitron). Proliferation was measured by the incorporation of [³H] thymidine into DNA during the final 12 h of incubation. The results are expressed as the stimulation index (S.I.): the ratio of the mean radioactivity (cpm) of the antigen to the mean

background (cpm) obtained in the absence of the antigen. Standard deviation was always <10% of the mean cpm.

Statistical Analysis: A one-way ANOVA test was used to compare independent values. $p < 0.05$ was accepted as statistically significant.

5

EXAMPLE 1

Inhibition of atherogenesis in genetically predisposed (Apo-E-deficient) mice by induction of nasal tolerance with low doses of the plaque associated molecules oxidized LDL, human Beta2GPI and HSP 65

10 The present inventors here demonstrate, for the first time, that nasal exposure to low doses of the plaque associated molecules oxidized LDL, Beta2GPI and HSP 65 provides induction of tolerance to the antigens, and significant inhibition of atherogenesis. Thus, nasal exposure to purified, oxidized human LDL, human Beta2GPI and recombinant mycobacterial HSP
15 65 were compared for their effectiveness in suppressing atherogenesis in Apo-E-deficient mice. 63 male 9-13 week old Apo E/C 57 mice were divided into 5 groups. In group A (HSP-65)(n=12) nasal tolerance was induced as described in Materials and Methods by administration of recombinant mycobacterial HSP 65 suspended in PBS (10 µg/mouse/10µl) for 5 days every
20 other day. In group B (H-oxLDL)(n=14) nasal tolerance was induced as described in Materials and Methods by administration of 10 µg/mouse/10µl oxidized purified human LDL, suspended in PBS, every other day for 5 days. Mice in group C (B2GPI)(n=13) received 10 µg/mouse/10µl human Beta2GPI per mouse, administered intranasally as described in Materials and Methods,
25 every other day for 5 days. Mice in group D (BSA)(n=12) received 10 µg/mouse/10µl bovine serum albumin (BSA) per mouse, administered intranasally as described in Materials and Methods, every other day for 5 days. Mice in group E (PBS)(n=12) received 10µl PBS per mouse, administered intranasally. Mice were bled prior to feeding (Time 0) and at the conclusion of

the experiment (End) for determination of lipid profile. Atherosclerosis was assessed in heart and aorta as described above, 8 weeks after the last feeding. Mice were weighed every 2 weeks during the experiment. All mice were fed water ad libitum and a normal chow-diet containing 4.5% fat by weight (0.02% cholesterol), up to the final antigen exposure, and then a "Western" diet until sacrifice.

Table 1: Inhibition of atherogenesis in Apo-E-deficient mice by intranasal administration of exceedingly low doses of plaque associated molecules

		HSP-65	H-oxLDL	H-B2-GPI	BSA	PBS	
Time 0	Weight (gr) (Mean±S.E)	22.6 ±0.8	22.3 ±0.5	22.3 ±0.7	21.8 ±0.7	21.7 ±0.5	P=0.833
	Chol (mg/dL) (Mean±S.E)	237 ±13	230 ±10	230 ±14	236±19	227±14	P=0.986
	TG (mg/dL) (Mean±S.E)	150 ±19	178 ±17	162 ±18	185±22	160±15	P=0.664
END	Weight (gr) (Mean±S.E)	26.8 ±0.9	28.2 ±1.0	29.2 ±1.5	25.5±1.0	26.3±1.3	P=0.157
	Chol (mg/dL) (Mean±S.E)	1181 ±114	1611 ±119	1601 ±125	1470±183	1606 ±181	P=0.197
	TG (mg/dL) (Median)	288	275	380	315	403	P=0.416
	Sinus Lesion (µm ²) (Mean±S.E)	44375 ±5437	43393 ±4107	46250 ±4486	120500 ±8746	128182 ±9102	P<0.001

Note: "Weight" is weight in grams; "Chol" is serum cholesterol and "TG" is serum triglycerides, expressed in mg/dL.

As can be seen from Figure 1, the results depicted in Table 1 demonstrate the strikingly effective inhibition of atherogenesis measured in the tissues of mice receiving nasal exposure to low doses (10 µg/ mouse) of the plaque associated molecules, compared to control mice exposed to sham antigen (BSA) or PBS. Furthermore, nasal tolerance is specific in its mode of protection: clearly, induction of nasal tolerance has no significant effect on other general parameters measured, such as weight gain, triglyceride or cholesterol blood levels. Thus, the antigenic plaque associated molecules oxidized LDL, Beta2GPI and HSP 65 are highly potent inducers of nasal

tolerance, with surprisingly low doses (10 µg/ mouse) and brief exposure (3 days) of significant (greater than 65%) and consistent protection from atherogenesis in these genetically susceptible Apo-E-deficient mice.

EXAMPLE 2

Superior inhibition of atherogenesis in genetically predisposed (Apo-E-deficient) mice by induction of nasal tolerance with HSP 65

The present inventors here demonstrate, for the first time, that nasal exposure to exceedingly low doses of the plaque associated molecule HSP 65 provides superior induction of tolerance to the antigen, and inhibition of atherogenesis. Thus, nasal exposure to a low dose and an exceedingly low dose of recombinant human HSP 65 were compared for their effectiveness in suppressing atherogenesis in Apo-E-deficient mice. 58 male 12-16 week old Apo E/C 57 mice were divided into 4 groups. In group A (HSP-65 high)(n=14) nasal tolerance was induced as described in Materials and Methods by intranasal administration of 10µg/mouse/10µl recombinant human HSP 65 suspended in PBS for 5 days every other day. In group B (HSP-65 low)(n=16) nasal tolerance was induced as described in Materials and Methods by administration of 1 µg/mouse/10µl recombinant human HSP 65 suspended in PBS every other day for 5 days. Mice in group C (BSA)(n=14) received 1 µg/mouse/10µl BSA per mouse, administered intranasally, every other day for 5 days. Mice in group D (PBS)(n=14) received 10µl PBS per mouse, administered intranasally. Mice were bled prior to feeding (Time 0) and at the conclusion of the experiment (End) for determination of lipid profile. Atherosclerosis was assessed in heart and aorta as described above, 8 weeks after the last feeding. Mice were weighed every 2 weeks during the experiment. All mice were fed water ad libitum and a normal chow-diet containing 4.5% fat by weight (0.02% cholesterol), up to the final antigen exposure, and then a "Western" diet until sacrifice.

Table 2: Superior inhibition of atherogenesis in Apo-E-deficient mice by intranasal administration of human HSP 65

		HSP65 10 μg/Mouse N=12	HSP65 1 μg/Mouse N=16	BSA 100 μg/Mouse N=11	PBS N=10	Statistics
End	Wt	28.4 ±1.0	26.9 ±0.9	27.7 ±0.5	28.7 ±0.7	P=0.363
	Chol	1073 ±65	1010 ±64	1009 ±74	1015 ±85	P=0.897
	TG	348 ±32	315 ±46	316 ±32	390 ±44	P=0.564
	Sinus Les. μm ²	22292 ±2691	17109 ±2053	54432 ±8201	47750 ±5779	P<0.05 Between HSP- 65 and PBS or BSA

Note: "Weight" is weight in grams; "Chol" is serum cholesterol and "TG" is serum triglycerides, expressed in mg/dL.

As can be seen from Figure 2, the results depicted in Table 2 demonstrate the superior effectiveness of inhibition of atherogenesis measured in the tissues of mice receiving nasal exposure to exceedingly low doses (1 μg/mouse) of HSP 65, compared to control mice exposed to sham antigen (BSA) or PBS. Furthermore, nasal tolerance is specific in its mode of protection: clearly, induction of nasal tolerance has no significant effect on other general parameters measured, such as weight gain, triglyceride or cholesterol blood levels. Thus, the antigenic plaque associated molecule HSP 65 is an extremely potent inducer of nasal tolerance, with even exceedingly low doses conferring significant (approximately 70%) protection from atherogenesis in genetically susceptible Apo-E-deficient mice, greatly superior to the protection achieved by induction of oral tolerance (30%; see US Patent Application No 09/806,400 to Shoenfeld et al filed Sept 30, 1999).

EXAMPLE 3

Superior suppression of specific anti-Beta2GPI immune reactivity in genetically predisposed (apo-E deficient) mice by intranasal administration of human Beta2GPI

Tolerance induced by mucosal exposure to plaque associated molecules may be mediated by suppression of specific immune responses to antigenic

portions of these plaque associated molecules. Lymphocyte proliferation in response to mucosal (nasal and oral) exposure to human Beta2GPI was measured in Apo-E-deficient mice. 9 male, 5 week old Apo E/C 57 deficient mice were divided into 3 groups. In group A (n=3) oral tolerance was induced with 100 µg/mouse Beta2GPI suspended in 0.2 ml PBS, administered by gavage, as described above, every other day for 5 days. In group B (n=3) nasal tolerance was induced with 10 µg/mouse Beta2GPI suspended in 10 µl PBS, administered intranasally as described above, every other day for 5 days. The mice in group C (n=3) received oral administration of 200 µl PBS every other day for 5 days. Immune reactivity was stimulated in all mice by immunization with human Beta2GPI as described above in the Materials and Methods section, one day after the last feeding. Ten days after the immunization lymph nodes were collected for assay of proliferation. All mice were fed normal chow-diet containing 4.5% fat by weight (0.02% cholesterol) and water ad libitum.

Table 3: Intranasal pretreatment with purified human beta2 GPI suppresses immune response to Human Beta2GPI in Apo-E-deficient mice

	PBS	H-β ₂ -GPI OT	H-β ₂ -GPI NT
S.I (Stimulation Index)	7.0±0.2	4.4±0.5	2.1±0.5

As can be seen from Figure 3, the results depicted in Table 3 demonstrate significant suppression of immune reactivity to human Beta2GPI antigen, measured by inhibition of proliferation in the lymph nodes of Apo-E-deficient mice. Lymphocytes from mice receiving intranasal exposure to low atherogenesis-inhibiting doses (10 µg/ mouse) of human Beta2GPI showed an exceedingly reduced stimulation index following immunization with Beta2GPI, as compared to orally exposed and control (PBS) mice. Since previous studies with induction of nasal tolerance have shown no significant

effect on other parameters measured, such as weight gain, triglyceride or cholesterol blood levels, or immune competence (see abovementioned Examples), these results indicate a specific suppression of anti-Beta2GPI immune reactivity. Thus, intranasal administration of the purified plaque associated molecule Beta2GPI is a superior method of attenuating the cellular immune response to immunogenic and atherogenic plaque associated molecules in these genetically susceptible Apo-E-deficient mice.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents, patent applications and sequences identified by their accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent, patent application or sequence identified by their accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition for prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, comprising, as an active ingredient, a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule, or pharmaceutical salts thereof, the composition further comprising a pharmaceutically acceptable carrier, the pharmaceutical composition being designed for mucosal administration.

2. The composition of claim 1, wherein said plaque associated molecule is selected from the group consisting of oxidized LDL, beta-2-GPI, HSP and derivatives thereof.

3. The composition of claim 1, wherein said antigenic portion of at least one plaque associated molecule is a naturally occurring molecule or a synthetic molecule.

4. The composition of claim 1, wherein the pharmaceutical composition is formulated for nasal, respiratory, otic and/or conjunctival administration.

5. The composition of claim 1, wherein said at least said antigenic portion of said at least one plaque associated molecule is selected so as to reduce immune reactivity to plaque components in the subject.

6. The composition of claim 1, packaged and identified for use in the prevention and/or treatment of at least one disorder selected from the group

consisting of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis.

7. The composition of claim 1, further comprising a therapeutically effective amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

8. An assay for determining the effect of mucosal administration of plaque components on atherosclerosis-related disease or condition, the assay comprising:

- (a) mucosally administering to a subject having an atherosclerosis-related disease or condition at least an antigenic portion of at least one plaque associated molecule and,
- (b) assessing at least one indicator of atherogenesis in said subject to thereby determine the effect of mucosal administration of said at least said antigenic portion of said at least one plaque associated molecule on the atherosclerosis-related disease or condition .

9. The assay of claim 8, wherein said plaque associated molecule is selected from the group consisting of oxidized LDL, beta-2-GPI, HSP and derivatives thereof.

10. The assay of claim 8, wherein said antigenic portion of said at least one plaque associated molecule is a naturally occurring molecule or a synthetic molecule.

11. A method of prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease,

stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, the method comprising mucosally administering a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule to the subject.

12. The method of claim 11, wherein said plaque associated molecule is selected from the group consisting of oxidized LDL, beta-2-GPI, HSP and derivatives thereof.

13. The method of claim 11, wherein said at least said antigenic portion of at least one plaque associated molecule is a naturally occurring or synthetic molecule.

14. The method of claim 11, wherein administration of said antigenic portion of said at least one plaque associated molecule is effected via nasal, respiratory, otic and/or conjunctival route.

15. The method of claim 11, wherein administration of said at least said antigenic portion of at least one plaque associated molecule reduces immune reactivity to said at least one plaque associated molecule in said subject.

16. The method of claim 11, wherein said at least said antigenic portion of said at least one plaque associated molecule is administered along with a therapeutically effective amount of a compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

1/2

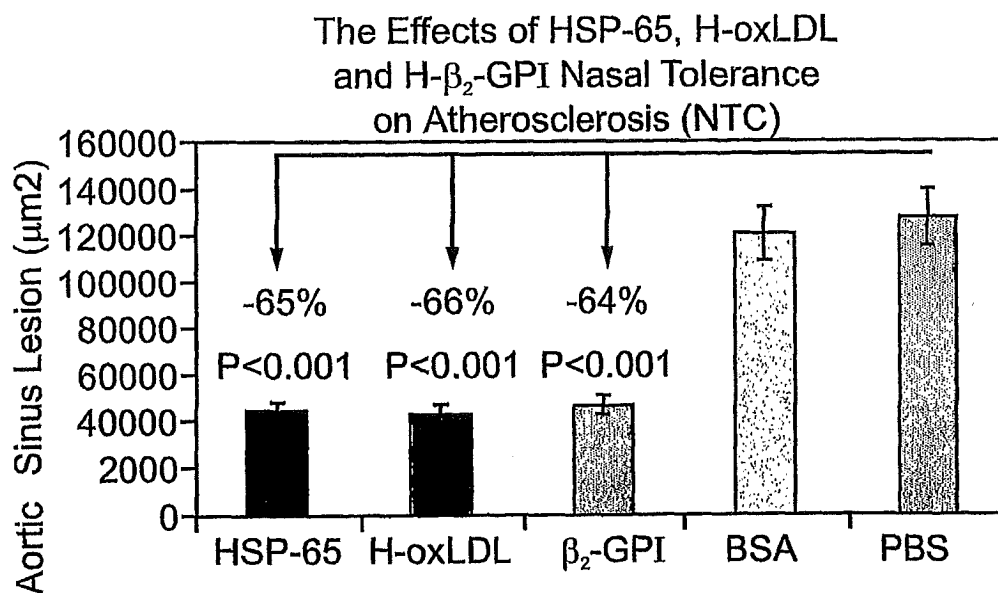


Fig. 1

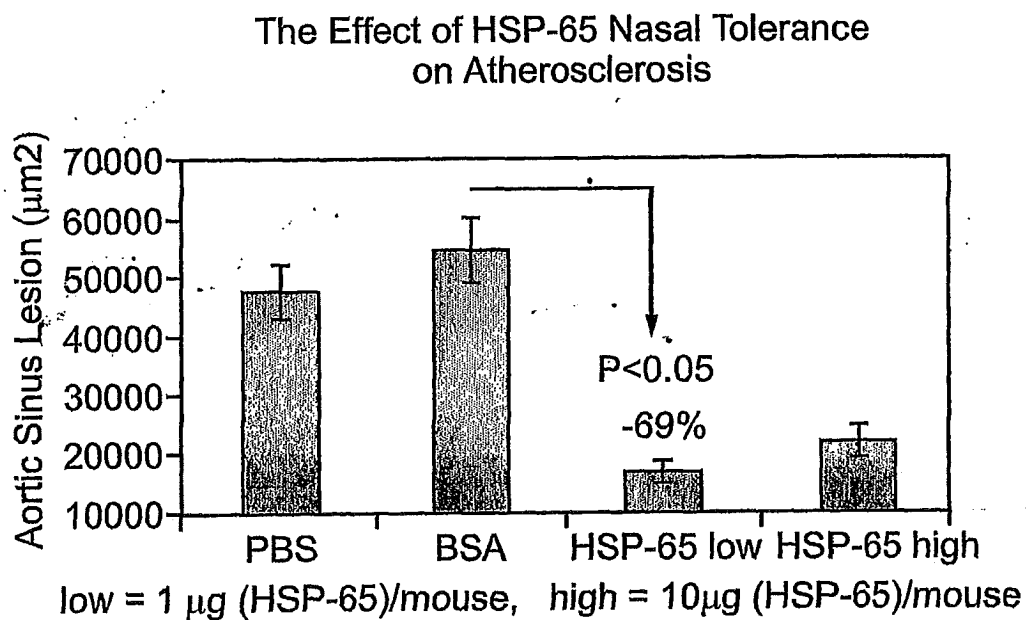


Fig. 2

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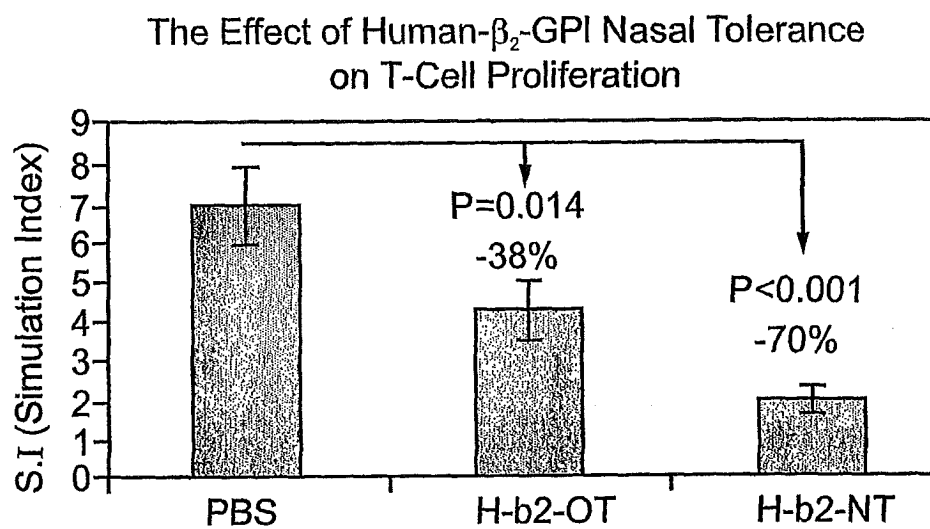


Fig. 3

EXHIBIT 11

Pathways for self-tolerance and the treatment of autoimmune diseases

Christopher C Goodnow

Antigen delivers both immunogenic and tolerogenic signals to lymphocytes. The outcome of antigen exposure represents a complex integration of the timing of antigen binding with signals from many other immunogenic and tolerogenic costimulatory pathways. A road map of these signalling pathways is only beginning to be charted, revealing the mechanism of action and limitations of current immunotherapeutic agents and the points of attack for new agents. Cyclosporin and tacrolimus interfere with tolerogenic signals from antigen in addition to blocking immunogenic signals, thus preventing active establishment of tolerance. Corticosteroids inhibit a key immunogenic pathway, NF κ B, and more specific inhibitors of this pathway may allow tolerance to be actively established while immune responses are blocked. New experimental therapies aim to mimic tolerogenic antigen signals by chronically stimulating antigen receptors with antigen or antibodies to the receptor, or aim to block costimulatory pathways involving CD40 ligand, B7, or interleukin 2. Obtaining the desired response with these strategies is unpredictable because many of these signals have both tolerogenic and immunogenic roles. The cause of autoimmune diseases has been determined for several rare monogenic disorders, revealing inherited deficiencies in tolerogenic costimulatory pathways such as FAS. Common autoimmune disorders may have a biochemically related pathogenesis.

Self-tolerance is an essential feature of the immune system, and works to protect tissue antigens from becoming targets of damaging immune responses during clearance of infection. The immune system normally exhibits exquisite specificity in distinguishing infectious antigens from self antigens. Vigorous antibody or T-cell responses are mounted against infectious antigens, whereas self antigens generally elicit only transient or weak responses even when incorporated into an infectious particle.

Adaptive immune responses start with the binding of antigen to antigen receptors on rare lymphocytes. The number and activity of these cells is then greatly expanded by clonal proliferation and differentiation. The response of individual lymphocytes is governed, however, by opposing immunogenic and tolerogenic signals, and the latter normally prevail for lymphocytes that bind self antigens. Disturbance in the natural balance between immunogenic and tolerogenic signals due to genetic factors can give rise to autoimmune disease. Progress in delineating these opposing signals provides opportunities to correct the primary disorder in autoimmune patients.

Counterbalancing immunogenic and tolerogenic signals

Two basic types of extracellular stimuli control lymphocyte growth and development (figure 1). The first is antigen signalling, through clone-specific antigen receptors. The second is costimuli, which encompasses a number of signals, through receptors that are not antigen specific. Importantly, particular antigen or costimuli

signals are rarely obligately immunogenic or tolerogenic. Their timing and the way they are integrated at key checkpoints in lymphocyte development determines how a lymphocyte responds. Strongly immunogenic costimuli can shift the balance to immunity in the face of strongly tolerogenic antigen signals, and strongly tolerogenic costimuli can over-ride strongly immunogenic antigen signals. Deciphering the molecular logic behind this signal integration is the central challenge facing clinical manipulation of tolerance and immunity.

Immunogenic and tolerogenic antigen signals

Antigens transmit signals to lymphocytes by binding to B-cell receptors (surface immunoglobulin on B cells), and to T-cell receptors (TCRs) on T cells. B-cell receptors and TCRs signal through a cascade of protein tyrosine kinases and protein-lipid phosphorylation. Antigen transmits immunogenic or tolerogenic signals to lymphocytes through these receptors. Continuous

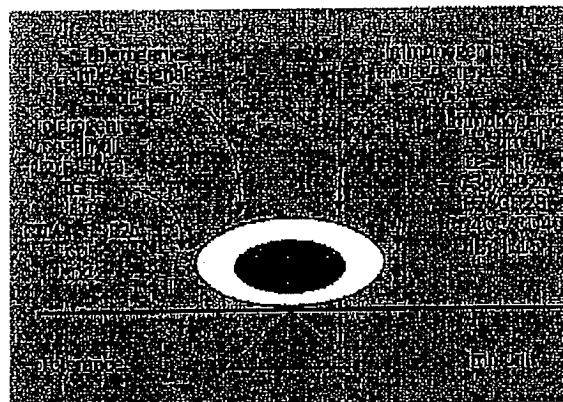


Figure 1: Schematic diagram illustrating the balance of immunogenic and tolerogenic signals affecting lymphocyte responses to antigen
LPS=lipopolysaccharide.

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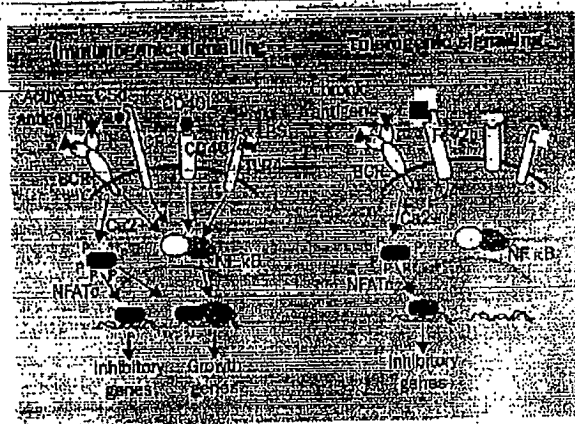


Figure 2: Biochemically distinct signals underpin immunogenic and tolerogenic responses to antigen in B lymphocytes

Immunogenic signalling occurs when antigen is encountered suddenly by mature B cells, and is augmented by co-clustering of complement C3d-receptor, CR2, and concurrent stimulation by CD40L from helper T cells or bacterial products such as lipopolysaccharide (LPS). One of the chief pathways activated by these signals is NFκB, a DNA binding protein family that moves to the nucleus once activated. In the nucleus, NFκB is pivotal to stimulating expression of many key B lymphocyte growth genes, promoting cell proliferation and antibody. The B-cell receptor also activates another DNA binding protein, NFATc, which moves to the nucleus after calcium-induced dephosphorylation and can work synergistically with NFκB. On its own, NFATc can activate inhibitory genes such as the inhibitory receptor CD72. Tolerogenic signalling occurs when antigen is encountered chronically, which results in inhibitory changes that diminish calcium signalling so that NFκB is no longer activated. Co-clustering of the receptor for IgG, FcγR2b, also inhibits immunogenic signalling to NFκB. Absence of costimuli such as CD40L or LPS is also critical to allow tolerogenic signalling to proceed in the absence of NFκB.

binding of antigen over several days, as is often the case for self antigens, usually transmits tolerogenic signals. By contrast, a sudden increase in receptor crosslinking, as occurs in most infections, tends to transmit immunogenic signals. Binding of antigen during immature lymphocyte formation in bone marrow or thymus, as occurs for many self antigens but few infectious antigens, tends to be tolerogenic.¹ Immunogenic signals are favoured when antigen is first encountered after lymphocytes have matured and reached the secondary lymphoid tissues, where infectious antigens tend to be trapped.

Immunogenic and tolerogenic antigens elicit different biochemical signals within lymphocytes² (figure 2). These biochemical differences provide opportunities to develop immunosuppressants that mirror these different signal patterns. In mature B lymphocytes, tolerogenic signalling by antigen elicits a smaller calcium response than immunogenic antigen. The calcium concentration achieved with tolerogenic signals is enough to activate the nuclear factor of activated T cells (NFATc) but insufficient to activate the nuclear factor kappa binding molecule (NFκB). NFATc and NFκB are DNA binding transcription factors that promote expression of different sets of genes. NFAT is essential for turning on lymphocyte inhibition as well as activatory genes, whereas NFκB is more purely immunogenic, because it is essential for inducing genes necessary for B and T cell proliferation and antibody production. As a result, a different pattern of gene expression is established by tolerogenic and immunogenic exposures to the same antigen.³

Deficiency of the NFκB transcription factor, c-rel, abolishes both T and B cells' immunogenic responses to antigen.⁴ The inherited immunodeficiency syndrome, X-linked agammaglobulinaemia, is caused by defects in Bruton's tyrosine kinase (BTK), an intracellular enzyme

that is essential for immunogenic signalling to NFκB by B-cell receptors.⁵ Tolerogenic signalling to antigen remains intact or enhanced in BTK-defective B cells. This selective role in immunogenic signalling might explain the powerful suppression of systemic lupus in NZB/W mice when defects in BTK are introduced by breeding.⁶ The selective role of the BTK/NFκB pathway in immunogenic signalling to antigen thus makes it an attractive target for new immunosuppressive drugs.

Immunogenic costimuli from microorganisms

Costimuli arise from many sources in the lymphocyte microenvironment. Perhaps the only purely immunogenic costimuli come from conserved components of infectious microorganisms. The lipopolysaccharide (LPS) moiety of bacterial cell walls and DNA rich in the dinucleotide, CpG from bacteria both activate the NFκB pathway in lymphocytes through surface receptors of the Toll-like receptor (TLR) family.^{7,8} (figure 2). These immunogenic costimuli also signal lymphocytes indirectly by activating antigen presenting cells—dendritic cells, macrophages, and B cells, to produce additional immunogenic costimuli such as the T cell activating cell surface protein B7 (CD80) and the inflammatory cytokine tumour necrosis factor alpha (TNFα). Bacterial adjuvants have been explored as experimental therapeutics to promote immunogenic responses to autoantigens on tumour cells but give rise to other undesirable inflammatory effects. Their effect may be more specifically emulated by activating dendritic cells bearing tumour antigens in vitro and giving these cells to the patient.

Costimuli from stressed and dying cells

Cell death through apoptosis occurs physiologically in healthy tissues without inflammation or immunogenicity. Engulfment of apoptotic cells by tissue macrophages, dendritic cells, or fibroblasts elicits signals through the phosphatidylserine receptor that promote synthesis of the tolerogenic cytokine, transforming growth factor beta (TGFβ; figure 3) and inhibit production of the immunogenic cytokine TNFα.⁹ By contrast, pathological cell death by necrosis links antigens with immunogenic costimuli. Necrotic cells, and antigens released from necrotic or stressed cells complexed with the heat-shock proteins, Hsp96 and Hsp70, activate dendritic cells to express immunogenic costimuli including B7 and TNFα.¹⁰⁻¹¹ In patients and animals models with developing neoplasms, increased production of these immunogenic costimuli through cell dysplasia and necrosis may account for the frequent detection of subclinical autoantibodies and for the less frequent paraneoplastic autoimmune syndromes. The latter might simply reflect rare clinical manifestations of common autoimmune responses to dysplastic tumour cell autoantigens, as a result of chance reactivity of the autoantibodies with a vital cell receptor. Likewise, cell stress and dysfunction in specific organs, such as the pancreatic beta cell, may be an immunogenic costimulus for autoimmunity.

Dual role of the complement system

Activation of the serum complement system by foreign cells or particles produces powerfully immunogenic costimuli, partly by covalently tagging the infectious antigens with the complement cleavage product C3d.¹² C3d signals immunogenically to B lymphocytes, through the complement C3d receptors, CR1 and CR2 (CD21

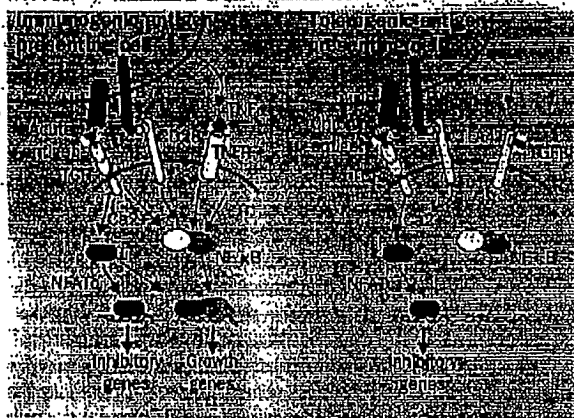


Figure 3: Biochemically distinct signals underpin immunogenic and tolerogenic responses to antigen in T lymphocytes

Immunogenic signalling occurs when antigen peptide and MHC complexes are encountered suddenly by mature T cells, and is augmented by concurrent stimulation by B7 molecules engaging CD28, or binding of TNF α . One of the chief pathways activated by these signals is NF κ B, a DNA binding protein family that moves to the nucleus once activated. In the nucleus, NF κ B is pivotal to stimulating expression of many key T lymphocyte growth genes, promoting cell proliferation and inflammatory cytokines. The TCR also activates another DNA binding protein, NFATc, which moves to the nucleus after calcium-induced dephosphorylation and can work synergistically with NF κ B. On its own, NFATc can activate inhibitory genes such as the death receptor ligand, FasL. Tolerogenic signalling occurs when antigen is encountered chronically, which results in inhibitory changes that diminish calcium signalling so that NF κ B is no longer activated. CD28 is downregulated and an inhibitory receptor for B7, CTLA4, is upregulated. Concurrent stimulation by TGF β inhibits expression of lymphocyte growth genes.

and CD35), when a C3d-tagged antigen causes clustering of these receptors with the B-cell receptors (figure 2). Complement components C1, C2, C4, and the CR1/2 complement receptors are also important for delivering tolerogenic signals, since inherited deficiencies of these elements in human beings and mice are associated with susceptibility to autoimmune disease. C1q deficiency leads to an inability to clear apoptotic cells efficiently, and this may either diminish the tolerogenic signals elicited by physiological cell corpses or allow them to become immunogenic.¹⁴

Dual role of the B7 system

Cell surface proteins of the B7 family, displayed on antigen presenting cells such as macrophages, dendritic cells, and B lymphocytes, deliver immunogenic costimuli to T cells by signalling through the CD28 and inducible costimulator (ICOS) receptors^{15,16} (figure 3). The B7.1 and B7.2 proteins are induced on antigen presenting cells by other immunogenic costimuli, such as LPS, necrotic cells, or immunogenic antigen receptor signals in B cells, creating a cascade of immunogenic signals. Immunosuppressive therapy aimed at blocking the immunogenic effects of B7.1 and B7.2, notably the recombinant protein antagonist CTLA4-Ig, has been shown to improve the symptoms of psoriasis.

B7/CD28 costimuli are tolerogenic in other contexts, notably in immature thymocytes where they enhance clonal deletion. The B7/CD28 pathway also promotes tolerance by signalling the formation of regulatory CD4⁺CD25⁺ T cells that may be required for tolerance to tissue antigens.¹⁷ B7.1 and B7.2 proteins also transmit tolerogenic signals to T cells by engaging another receptor, CTLA4, that is present at very low levels in resting T cells and substantially increased by chronic antigen signals¹⁴ (figure 3). The importance of CTLA4 as a brake to the system is shown by the lethal inflammatory

and lymphoproliferative disorder that occurs in CTLA4-deficient mice, and by the augmented autoimmune responses to melanoma antigens that occur when CTLA4 is blocked with antibodies.

Dual role of TNF α family of proteins and receptors

Activation of T cells and other innate or adaptive immune cells elicits an important and growing class of immunogenic and tolerogenic costimuli related to the cytokine, TNF α . TNF α itself has a pleiotropic effect on immune responses and inflammatory cells.¹⁸⁻²⁰ In some contexts, TNF α promotes self-tolerance and CD8 T cell deletion, whereas in others TNF α promotes T cell activation and autoimmune disease. Inherited deficiencies in TNF α or its receptors in mice results in poor cytotoxic T-cell-mediated resistance to certain viruses and inability to form follicular dendritic cells needed for humoral immunity. Symptoms of rheumatoid arthritis improve after blocking TNF α with antibodies or recombinant protein antagonists, indicating that production of this cytokine by T cells in the synovium has a key inflammatory role.²¹

CD40-ligand (CD40L) and Fas-ligand are two proteins related to TNF α with essential regulatory functions. Both are membrane-bound proteins displayed on T cells following T-cell receptor signals. CD40L engages its receptor, CD40, on B cells and dendritic cells to activate immunogenic responses through the NF κ B pathway.²² The importance of CD40L as an immunogenic costimulus is shown in children and mice with inherited CD40L deficiency, the X-linked hyper-IgM syndrome, where there is an absence of IgG antibody responses and defective T-cell immunity. Experimental therapies based on blocking the immunogenic effects of CD40L on B cells and dendritic cells with antibodies showed spectacular promise in animal models, notably achieving long-term allograft tolerance in primates.²⁴ Clinical trials in human beings have been suspended, however, because of thromboembolic complications in a subset of participants.

CD40L also seems to have an important tolerogenic role, since CD40L-deficient children are also commonly affected by autoimmune disease. CD40L is needed as a tolerogenic signal for B cells to increase expression of Fas (CD95), the receptor for FAS-L.²⁵ FAS itself transmits a potent tolerogenic costimulus by triggering the death and deletion of self-reactive B and T lymphocytes. The importance of the FAS pathway is seen by the systemic Autoimmune Lymphoproliferative Syndrome (ALPS) in human beings and mice with inherited deficiencies in FAS-L, FAS, or the downstream protease Caspase-10.²¹⁻²⁶

Inhibitory co-receptors

This is a rapidly growing class of receptors that transmit inhibitory or tolerogenic costimuli to lymphocytes functions by recruiting protein tyrosine or lipid phosphatases. The prototype for this family is the low affinity receptor for IgG, Fc γ R2b, on B cells.²⁷ Antigen and antibody complexes cause the antigen receptors to cluster with Fc γ R2b, preventing B cell activation by otherwise immunogenic antigens and, instead, triggering death and deletion of the B cells (figure 2). This mechanism is believed to explain the tolerogenic effect of anti-RhD prophylaxis, in which small amounts of IgG anti-RhD antibodies given to Rh-negative mothers prevent maternal antibody responses to fetal RhD antigen.

Cytokines

Cytokines deliver both immunogenic and tolerogenic costimuli to lymphocytes. This balance is well illustrated by interleukins 2, 7, and 15.^{21,24-26} All three signal T and B cells through multisubunit receptors that share a common gamma chain (γ c). Inherited deficiency of the γ c subunit accounts for X-linked Severe Combined Immunodeficiency, characterised by lack of naïve or memory T and B cells. IL-7 delivers essential costimuli through γ c that promote naïve T and B cell formation in thymus and bone marrow and promote survival of naïve T cells in the lymph nodes. Similarly, growth and persistence of memory CD8 T cells is promoted primarily by IL-15. By contrast, the essential function of IL-2 *in vivo* is to deliver a tolerogenic costimulus, despite its original discovery in tissue culture as a so-called T-cell growth factor. Mice lacking IL-2 or the unique IL-2 receptor alpha subunit develop a severe T cell lymphoproliferative disease with numerous autoantibodies. IL-2 sensitises T cells to receive tolerogenic signals by the Fas receptor system, and may also be required to sustain a tolerogenic subset of CD4+CD25+ regulatory T cells.

Transforming growth factor beta (TGF- β) delivers an important tolerogenic signal to lymphocytes, and mice lacking this cytokine rapidly develop a lethal syndrome of lymphocyte hyperactivity and autoantibodies.²⁷ TGF- β inhibits the entry of lymphocytes into the cell cycle, and thus might establish a high tolerogenic threshold against which immunogenic signals from antigen and costimuli must work to initiate lymphocyte responses. The early response to immunogenic antigen is differentiated from tolerogenic antigen responses in part by rapid downregulation of inhibitory transcription factors in the former.²⁸ TGF- β seems likely to establish these inhibitory factors in quiescent and tolerated lymphocytes. TGF- β production by macrophages is induced by recognition and engulfment of cells that have died by physiological (non-inflammatory) apoptosis.²⁹ Macrophages, dendritic cells, and T cells making TGF- β seem to promote tolerance to self and foreign antigens in the eye, lung, and gut.^{30,31} Linking antigen signals with TGF- β signals may be the basis for the experimental phenomenon of oral tolerance. Clinical trials are underway aimed at preventing type 1 diabetes or ameliorating multiple sclerosis by inducing oral tolerance to pro-insulin or myelin basic protein.

Integration of tolerogenic and immunogenic signals at different steps in the immune response

Integration and timing of antigen signals and costimuli occur at numerous checkpoints in lymphocyte development. These checkpoints are placed all along the developmental pathway, from those that delete newly formed B or T cells in the bone marrow and thymus through to those that abort the formation of terminally differentiated plasma cells or killer cells. Lymphocytes integrate antigen signals and costimuli very differently from one checkpoint to another, because expression of receptors and their intracellular response machinery change during development. The multiplicity of checkpoints exists presumably for two main reasons. First, no single mechanism can adequately ensure tolerance to all self antigens. Second, the existence of multiple mechanisms balances the need for tolerance against the need to use cells that crossreact between self and foreign antigens for rapid immunity against infection.³⁴

Clonal deletion in central lymphoid tissues

In the bone marrow and thymus, antigens that cluster antigen receptors rapidly and avidly—which would be immunogenic for a mature lymphocyte—are almost exclusively tolerogenic for newly formed B and T cells.³² The basis for the tolerogenic response of immature lymphocytes seems to be a result of many things: differences in the second messengers elicited by antigen receptors in immature cells, differences in the set of genes that can be triggered by second messengers, and presence of tolerogenic costimuli in the bone marrow and thymus microenvironments. Immature thymocytes are triggered to die even when antigen signals are linked with costimuli such as B7/CD28 that would be immunogenic to mature T cells. In immature B cells, continuous B-cell receptor engagement with strongly crosslinking self antigens, such as DNA or surface antigens on haematopoietic cells, delivers a tolerogenic signal that immediately arrests the cell's maturation and leads to clonal deletion within 1–3 days. Some of these arrested cells reach the spleen before dying, but they are extraordinarily refractory or anergic to immunogenic costimuli such as LPS and CD40. Particular combinations of immunogenic costimuli, such as CD40 and IL-4 from helper T cells, may be able to over-ride the powerfully tolerogenic signals from self antigen in these situations and break tolerance at this point.

Only a subset of self antigens are nevertheless present in sufficient quantity in the thymus and bone marrow to trigger clonal deletion. There is simply not enough antigen to signal deletion for most clones which recognise antigens present in trace quantities in the circulation or which are restricted to other tissues, such as the pancreatic islets, the brain, or the thyroid. Other mechanisms normally ensure tolerance to these antigens.

Clonal anergy

Self antigens that are present in lesser amounts in the bone marrow or thymus, or that cluster antigen receptors less avidly, can signal repeatedly to B and T cells without attaining the threshold needed to trigger arrest and death.³³ This constant "tickling" of antigen receptors by self antigens nevertheless transmits tolerogenic signals, activating feedback mechanisms that render the cell more refractory or anergic to immunogenic antigen signals. Anergy mediates B cell tolerance to self DNA and chromatin, and CD4 T cell tolerance to systemic and organ-specific antigens. In both B and T cells, anergy seems to involve a selective weakening of the connections between antigen receptors and the NF κ B and JNK intracellular signalling pathways. Signalling through other intracellular pathways such as NFAT remains intact, so that a different set of tolerogenic genes is induced and immunogenic cell growth genes controlled by NF κ B and JNK are not called into action. The weakening of connections to NF κ B and JNK raises the threshold of immunogenic signalling needed to trip a self-reactive cell into multiplication. In B cells, a sudden burst of very avid antigen receptor clustering, or strong signals from LPS or CD40, allow sufficient signalling to the NF κ B pathway to break anergy and drive the cell growth cycle.

Clonal deletion and regulation in peripheral lymphoid tissues

In addition to anergy, a series of peripheral deletion mechanisms catch self-reactive cells that reach the spleen, lymph nodes, and other organs.³⁵ These peripheral tolerance checkpoints act by shortening

lymphocyte lifespan, inhibiting lymphocyte migration and recirculation, or causing rapid cell death in germinal centres or liver. These peripheral processes are for the most part poorly understood in biochemical terms, with the exception of the peripheral elimination of autoreactive B and T cells through the Fas cell death pathway.^{31,32,33}

Pathogenesis of autoimmune diseases

How does autoimmune disease arise? Given the range of self-tolerance processes, and the difficulty eliciting or maintaining autoimmune responses by deliberate means (for example in medical and veterinary efforts to achieve immunological castration or castration), it is reasonable to ask how tolerance to one or more self antigens fails in many people. The reason is as yet unknown, except for the rare patients with inherited monogenic disorders such as ALPS and X-linked hyper-IgM.

Most of the common autoimmune diseases also have an important inherited element, contributing as much as 50% of the population risk, and particular types of autoimmune diseases thus cluster in families. This inherited susceptibility is nevertheless complex involving combinations of many different gene alleles.³⁴ The strongest contributions are made by particular haplotypes of the major histocompatibility complex (MHC) and specific HLA alleles within the MHC, whose products present antigen peptides to T cells. Exactly how particular MHC alleles predispose to autoimmunity is not yet established, and one can hypothesise too much or too little presentation of particular antigens by products of susceptible HLA alleles. Correlations between autoimmune susceptibility and many other chromosomal regions have been found in human beings and mice, but the complexity of the inheritance pattern has made it challenging to identify the non-MHC susceptibility genes.

Four basic kinds of defect may potentially give rise to autoimmune disease, either alone or in combination. A central challenge for clinical immunology will be to define which of these faults actually applies for individual patients, since the nature of the deficit will determine the success or failure of emerging therapeutic strategies.

Insufficient tolerogenic signalling from antigen

In order for deletion, anergy, or regulation to be triggered by tolerogenic signalling through antigen receptors, a sufficient number of receptors must be engaged on self-reactive cells. Autoantigens that are only present in trace amounts in the lymphatic tissues will not achieve this signalling threshold on any but the very highest affinity clones. If the autoantigen is highly expressed in extralymphatic sites, as is the case for insulin, thyroglobulin, myelin proteins, skin basement proteins, and type 2 collagen, these concentrated depots of autoantigen might suddenly deliver an acute immunogenic stimulus to self-reactive cells that chance to migrate into these sites. This situation seems to be the case for B cells and some CD8 T cells.^{35,36} For CD4 T cells recognising such antigens, there seems to be some autoantigen encountered in lymphatic sites that might induce anergy and regulatory cells.³⁴

Several susceptibility genes for type 1 diabetes may act by further diminishing this already limiting pathway for tolerogenic autoantigen presentation. Diabetes-susceptible MHC Class II alleles in human beings and mice seem less efficient at presenting antigens, potentially explaining the heightened risk of autoimmunity in individuals who are homozygous for these alleles.³⁴ A

variant allele of the insulin gene associated with type 1 diabetes susceptibility is expressed at lower levels in the thymus, potentially lessening presentation and education of regulatory T cells to this antigen.³⁷

If the primary lesion in individuals susceptible to type 1 diabetes and other organ-specific diseases is simply one of inadequate tolerogenic signals from the target self antigens, then delivering more of these antigens in a tolerogenic form is a rational strategy. Obviously, this approach has the risk of inducing autoimmunity if the self antigen is delivered in an immunogenic form in some individuals, either due to the way the antigen is presented, to presence of immunogenic costimuli, or to presence of primed or memory lymphocytes that may be more refractory to tolerogenic signals. A better understanding of the molecular integration of tolerogenic and immunogenic signals may be critical to the success of specific vaccines against diabetes and other autoimmune diseases.

Insufficient tolerogenic signals from autoantigen might also explain shortcomings of the immunosuppressive drugs, ciclosporin and tacrolimus (FK506). These drugs block the calcium/calcineurin/NFAT signalling pathway. This pathway is continually activated by self antigen in anergic B and T cells, and is important for inducing tolerogenic costimuli on lymphocytes such as CD72 and FAS-L. Interference with these actively tolerogenic signals might explain the systemic autoimmune disorders that can occur after cessation of the drug, and might account for the inability to achieve long-term allograft acceptance with these agents.³⁸ The presence of circulating autoantibodies may compound autoimmunity in systemic lupus by blocking the presentation of tolerising autoantigens to B cells.³⁹

Too much immunogenic signalling from antigen

Sudden presentation of viral or bacterial antigen in a highly crosslinked, immunogenic form, and associated with immunogenic costimuli produced by the infection, can provoke immune responses from T or B cells that crossreact with the microbial antigen and a self antigen. In animal models, this route can activate ignorant T and B cell clones that, through a combination of lower affinity receptors and limiting self-antigen presentation, had not received appreciable tolerogenic signals.^{39,40,41,42} Moreover, if the immunogenic antigen stimulus is very strong, such as occurs with highly multimeric forms of antigen for B cells, the stimulus can overcome strongly tolerogenic antigen signals to break anergy^{43,44} or over-ride clonal deletion.⁴⁵ Whereas a microbial trigger is postulated to be the cause of a number of common autoimmune diseases, perhaps the best established clinical example is the immunopathological damage of heart valves by antibodies that crossreact between valvular antigens and streptococcal M protein.

Interestingly, the self-reactive components of crossreactive responses are usually transient and lack memory in most experimental and practical situations in which tolerance is transiently broken by immunogenic delivery of self and foreign antigens. This phenomenon is a longstanding problem for medical and veterinary efforts to achieve immunocontraception and immunocastration, in which the autoantibody titres to pregnancy or sex hormones fall prematurely in the face of heightened titres to the foreign carrier proteins. Susceptibility to full-blown autoimmune disease might therefore require that a crossreactive trigger be coupled with deficits in the tolerogenic costimuli that normally create an inhibitory feedback on self antigen responses.

Deficiency of tolerogenic costimuli

Many of the rare systemic autoimmune disorders that are inherited as monogenic traits in human beings and mice arise from deficiencies of tolerogenic costimuli. The clearest example is human autoimmune lymphoproliferative syndrome (ALPS), which results from partial or complete deficiency in signalling by the death receptor FAS.²¹ Similarly, deficiency of CD40L in X-linked hyper-IgM syndrome is commonly accompanied by autoimmune disorders that might reflect the need for CD40L to induce Fas on self-reactive B cells.²² The monogenic autoimmune disorders listed above are clinically distinct from the common forms of autoimmune disease, but they illustrate the essential and non-redundant role of tolerogenic costimuli as brakes on autoimmunity. Common autoimmune disorders probably arise from collections of more subtle gene variants that collectively diminish the same tolerogenic pathways. In support of this notion, the type 1 diabetes susceptibility gene in the NOD mouse, *Idd3*, seems to be a variant form of IL-2 that may reduce the in-vivo efficacy of this tolerogenic costimulus.²³

Too much Immunogenic costimuli

There are many artificially engineered animal models where overexpression of immunogenic costimuli predisposes to autoimmune disease. For example, mice that overexpress TNF α , B7.1, IL-2, or IL-4 on pancreatic islet β -cells are predisposed to type 1 diabetes.²⁴ Cell death by necrosis releases antigens complexed with immunogenic costimuli, notably the heat-shock proteins HSP70 and HSP96, and necrotic cells activate dendritic cells. An increase in these tolerogenic immunogenic costimuli might explain the immunogenicity of dysplastic tumours that are commonly manifested by the appearance of subclinical autoantibodies to tumour antigens and by paraneoplastic autoimmune syndromes. Along similar lines, the inability to clear dead cells or chromatin might provoke systemic lupus in people with complement C1q deficiency.¹⁴

Targets for current and future therapy of autoimmune disease

The unfolding of the human genome project will accelerate assembly of a molecular map of immunogenic and tolerogenic signalling pathways. Translating this knowledge into cures for common autoimmune diseases will involve researchers addressing two key challenges. First, we must develop ways to diagnose the underlying cause of autoimmune disease in individual patients. There is probably little to be gained by giving an exogenous source of tolerogenic costimuli such as TGF- β or Fas-ligand to patients with an underlying problem further downstream in the receptors or signal-transduction pathways for these molecules. Methods for obtaining a genetic fingerprint of thousands of immunologically relevant genes will soon become available, and these might provide a way to shortlist the likely pathogenic deficits in individuals. Confirmation will probably require diagnostic biomarkers or specific assays for discrete immunogenic or tolerogenic pathways that can be done on blood samples.

The second critical element is development of protein or small molecule therapeutics that target critical pathways, either augmenting tolerogenic pathways or blocking immunogenic ones. Some of the best current agents for treating systemic autoimmune diseases, such as glucocorticoids, chloroquine, and gold compounds, seem to work by blocking the immunogenic NF κ B

pathway.²⁵ Improvements on these agents depend on narrowing the action to specific subsets of lymphocytes, and avoiding the undesirable metabolic effects of glucocorticoids. To cure fully developed autoimmunity, drug targets will need to come from understanding why memory T and B cells are more refractory to tolerogenic signals and why they are less dependent upon immunogenic costimuli.

Engineered proteins and antibodies aimed at blocking specific immunogenic costimuli upstream of NF κ B, notably antibodies against TNF α ,²⁶ CD40L,²⁷ and the blockers of B7 ligands of CD28, have shown great promise in mouse models and in clinical trials as agents to treat rheumatoid arthritis or establish transplantation tolerance. These strategies may be most effective in individuals with healthy tolerogenic signalling, such as patients undergoing organ transplantation, where the underlying defect is known to be an excess of immunogenic antigen and immunogenic costimuli. In this case, temporarily blocking the immunogenic signals selectively should allow tolerogenic antigen and costimuli to establish an active, reinforcing state of tolerance that persists when blocking therapy is stopped. However, if inherited deficits in tolerogenic signalling prevent restoration of tolerance during a brief window of blocking therapy, it will be necessary to continue the immunogenic blockers for long periods, even though there are many complications associated with long-term immunosuppression.

An attractive notion is the idea of so-called negative vaccines; vaccines that could deliver specific antigens in a way that augments tolerogenic rather than immunogenic signalling. In animal models, delivering low amounts of antigen by the mucosal route, either ingested or nasally, can act as a potent tolerogen. This process might perhaps work by linking the antigen with the tolerogenic costimulus, TGF- β , which features in mucosal immune responses.^{31,32} The first clinical trial of oral tolerance was unsuccessful, pointing to the need to understand better the mechanisms involved and to develop ways to achieve more reliable linkage between tolerogenic antigen and suitable tolerogenic costimuli. Likewise, rational molecular strategies are needed to improve the success rate of empirical regimes for desensitising allergic reactions to pollens and venoms and to restore tolerance to blood products such as clotting factor VIII.

The one shining example of a successful tolerogenic vaccine is the prevention of erythroblastosis fetalis in Rh-antigen incompatible pregnancies by giving small amounts of anti-RhD antibody. The antibody converts an immunogen (fetal red cells) into a tolerogen by recruiting a tolerogenic costimulus, Fc γ R2b. The wide success and cost-benefit of this simple method is an example of how it should become possible to shift the balance back towards tolerance in an antigen specific way for many autoimmune diseases. The key lies in understanding the molecular interplay between immunogenic and tolerogenic pathways and having a way to forge the desired tolerogenic connections in specific lymphocyte clones.

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EXHIBIT 12

Oral tolerance and inflammatory bowel disease

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Purpose of review

Oral tolerance refers to the ability of the mucosal immune system to actively inhibit systemic immune responses to fed antigens. Recently, clinical trials have used oral tolerance as a therapy for certain chronic inflammatory and autoimmune diseases such as multiple sclerosis and type 1 diabetes. Inflammatory bowel disease is now widely thought to be caused by the breakdown of oral tolerance through a combination of genetic and environmental factors. Therefore, it seems incongruous that clinicians would try to use oral tolerance therapy to alleviate the symptoms of inflammatory bowel disease. Yet, armed with the results of select animal models, trials have begun for oral tolerance therapy for Crohn's disease. This review will outline the recent advances in understanding oral tolerance, explore the relation between oral tolerance and inflammatory bowel disease, and comment on the likelihood of successful oral tolerance therapy for inflammatory bowel disease.

Recent findings

The results of an oral tolerance trial in Crohn's disease patients in Israel have shown some promising results, whereas the results of studies of experimentally induced oral tolerance in patients with inflammatory bowel disease from the authors' laboratory have shown that feeding a neoantigen in an attempt to induce oral tolerance is not successful in patients with inflammatory bowel disease.

Summary

The fundamental difference in the mechanisms of oral tolerance in mice and humans requires a more focused effort to understand the human mucosal immune system before oral tolerance therapy for autoimmune and chronic inflammatory disorders reaches its full potential.

Keywords

Crohn's disease, inflammatory bowel disease, oral tolerance

Introduction

Recent studies in both mouse models and human tissues have suggested that inflammatory bowel disease (IBD) is a consequence of the breakdown of normal mucosal tolerance. IBD patients have elevated serum antibodies against dietary antigens [1]. Tolerance to normal flora seems to be broken in IBD patients, which suggests active, not suppressed, immune responses against luminal antigens [2–4]. Therefore, the study of oral tolerance can have a significant clinical impact on IBD management. Although the role of CD4⁺ T cells in the mechanism of murine oral tolerance has been well defined, the contributions of CD8⁺ T cells, dendritic cells, and other cell types are currently being elucidated. Furthermore, no understanding of the mechanism of oral tolerance in humans exists. Recent work from Rescigno's [5••] lab has implicated an intestinal epithelial cell-derived cytokine that drives the mucosal dendritic cells to secrete antiinflammatory cytokines, contributing to the state of controlled inflammation that is the hallmark of the normal bowel. This cytokine was reduced or absent in intestinal epithelial cells (IECs) from five of seven Crohn's disease patients. Our laboratory [6,7] has published studies showing the absence of a molecule called gp180 from the epithelial cells of IBD patients and that experimental oral tolerance, in both Crohn's disease patients and ulcerative colitis patients in remission, could not be achieved.

These works highlight the relation between oral tolerance, intestinal homeostasis (controlled inflammation), and IBD and may give further insight into the mechanisms of each.

Oral tolerance

The term 'oral tolerance' refers to the active nonresponse to dietary antigens and commensal enteric bacteria or substances administered orally. Given that the systemic immune system must discriminate between self and non-self, the mucosal immune system must discriminate between potentially harmful pathogens and harmless luminal antigens. It has been suggested that the failure to do this results in food allergies and specific food intolerance such as celiac disease (response against food antigens) and Crohn's disease (response against bacterial flora) [8,9]. The mechanisms of oral tolerance induction have been extensively studied in murine systems. Repeated low-dose feeding results in a T cell-mediated suppression of immune responses. Multiple subtypes of regulatory T cells have been identified that involve the secretion of interleukin-10, interleukin-4, and transforming growth factor- β [10–12]. A single high-dose feeding results in T cell anergy or

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Abbreviations

IBD inflammatory bowel disease
IEC intestinal epithelial cell
KLH keyhole limpet hemocyanin

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deletion [13]. Aside from what is known about experimentally induced oral tolerance, there are many other properties of the gut-associated lymphoid tissue that contribute to intestinal homeostasis and the generally suppressive nature of the mucosal immune system.

Regulatory CD8⁺ T Cells in oral tolerance and inflammatory bowel disease

Although experimental oral tolerance does not require the presence of CD8⁺ T cells, other studies show that CD8⁺ T cells can have a suppressor phenotype [14–18]. We have shown that coculturing CD8⁺ T cells with IECs gives rise to a population of CD8⁺ cells with a suppressor cell phenotype [6]. This does not occur in cocultures with IECs of IBD patients. Recently, we have shown that there is an oligoclonal expansion of regulatory CD8⁺ T cells following coculture with normal IECs and that these cells are deficient in IBD patients [19,20]. Because CD8⁺ T cells were shown to be unnecessary in experimental oral tolerance in murine systems, this might be a fundamental difference in the mechanism of human and murine oral tolerance [14].

Dendritic cells

Recently, progress has been made in understanding the role of dendritic cells in mucosal tolerance. One of the first suggestions that dendritic cells play a role in tolerance was the use of Flt3 ligand in mouse models of oral tolerance induction [21]. In this model, a concurrent increase in dendritic cell populations in the intestine and experimental oral tolerance was observed. It was later shown that dendritic cells could express tight junction proteins and intercalate their dendrites between intestinal epithelial cells to sample luminal antigens without perturbing the mucosal barrier [22]. Furthermore, intestinal dendritic cells contain components of commensal bacteria [23^{*}]. Investigators have now identified an IEC-secreted cytokine, thymic stromal lymphopoietin, that matures dendritic cells into 'mucosal dendritic cells' that secrete interleukin-10, not interleukin-12, and help promote T_H2 maturation [5^{**}]. Interestingly, in a small survey of Crohn's disease patients, five of seven patients had undetectable amounts of this cytokine. Although the exact significance of this result is unknown, it does suggest that dendritic cells might play an important role in human oral tolerance and, by inference, IBD.

Pathogen recognition in the gut

Familial patterns of IBD suggest that there is a strong genetic component. In a genome-wide screen, genetic linkage analyses implicated *NOD2* as a predisposing genetic factor in Crohn's disease [24,25]. *NOD2* has been identified as a pathogen recognition molecule; *NOD2* recognizes a specific motif of bacterial peptidoglycan, muramyl dipeptide [26–28]. Interestingly, *NOD2*-deficient mice are susceptible to bacterial infection exclusively by the oral route. It has been recently shown that *NOD2* is required for

the expression of a subgroup of intestinal antimicrobial peptides known as cryptdins [29^{**}]. The *NOD2* mutations that are found in Crohn's patients are generally loss-of-function mutations, implying that for some patients with Crohn's disease, at least, the initial defect might be the lack the production of these defensins. Gain-of-function mutations also exist, however; these polymorphisms result in constitutive ligand-independent nuclear factor- κ B activity, which results in uncontrolled inflammation.

NOD2 is not the only clue that bacterial recognition might trigger colonic inflammation. The lipopolysaccharide-recognizing toll like receptor-4 has been shown to be over-expressed in the gastrointestinal tract in patients with Crohn's disease and ulcerative colitis and in models of murine colitis [30,31]. The recent findings of these bacterial recognition defects and their association to human disease are further evidence that IBD might be caused by a lack of suppression or tolerance to gut microflora [32^{**}].

Oral tolerance therapy in mouse models of human disease

Aside from understanding the mechanisms involved in mucosal tolerance, the idea that oral tolerance could be used to downregulate unwanted immune responses has been gaining popularity. In 1986, the first study was published showing that feeding soluble collagen to mice could inhibit collagen-induced arthritis [33]. Soon after that, other workers used a similar approach to inhibit other murine disease models such as experimental autoimmune encephalomyelitis, the model for multiple sclerosis, as well as models for rheumatoid arthritis, tissue graft rejection, autoimmune uveitis, and type I diabetes [34–40]. Generally, both prophylactic and therapeutic approaches for disease intervention have been very effective.

In studies of oral tolerance therapy for murine colitis, colonic proteins were fed to mice after trinitrobenzene sulfonic acid-induced colitis [41,42]. In this model, trinitrobenzene sulfonic acid is intrarectally administered to a C57/BL10 or SJL/J mouse [43]. The resulting colitis resembles the transmural lesions seen in Crohn's disease and is attributed to an interleukin-12-induced T_H1 response [44]. One possible significant difference between this model and human IBD is that this mouse strain can be tolerized to fed antigens. As mentioned below, studies from our laboratory suggest that IBD patients cannot become orally tolerized to fed antigens. Therefore, it might be more reasonable to expect an oral tolerance response to fed antigens, even colonic proteins, in these mice than in IBD patients.

Oral tolerance in humans

Armed with the effective use of oral tolerance in mouse models of inflammatory and autoimmune diseases, investigators started similar trials in humans. Unfortunately,

the data on the whole do not show particular efficacy [45,46].

The reasons why oral tolerance therapy in humans is not as successful as in mice are unclear. It is possible that the oral tolerance response in humans, using the doses and feeding schedule used in the trials, is not optimal. The optimal oral tolerance response in humans has not yet been fully explored. The first published study showed that repeated feeding of 50 mg of keyhole limpet hemocyanin (KLH), a neoantigen, resulted in a downregulation of T cell responses to subsequent subcutaneous administration of the antigen [47]. In contrast to murine oral tolerance, B cell responses were not inhibited. This study suggested that although the phenomenon of oral tolerance might be shared between species, the mechanisms of tolerance seem to differ.

Our laboratory has previously shown that epithelial cells from both involved as well as uninvolved mucosa of IBD patients lack a molecule called gp180 that is normally expressed on IECs and trophoblasts and has been found to bind to CD8 and associate with CD1d [7,48]. This complex interacts with the TcR/CD8 coreceptor complex on regulatory T cells [49]. To see whether gp180 is necessary for oral tolerance induction, we performed the human oral tolerance protocol in 16 patients with Crohn's disease and ulcerative colitis and more than 30 normal control individuals [50*]. We first found the lowest dose of KLH required for effective oral tolerance (50 mg) in normal control individuals before conducting our study. We found that whereas all the normal control subjects tolerized, 6 of 8 Crohn's disease patients and 7 of 8 ulcerative colitis patients did not tolerize to KLH. In fact, given that T cell responses increased even after the feeding alone, it seemed that feeding the antigen primed the systemic immune response in the ulcerative colitis patients. We concluded that patients with IBD seem to have defective oral tolerance responses and that this correlates with the lack of gp180 expression.

Both Crohn's disease and ulcerative colitis seem to have genetic determinants, Crohn's disease being more closely associated with genetic factors than ulcerative colitis [51]. Therefore, we also studied the oral tolerance responses of nonaffected family members from multiplex IBD families. In this study, 5 of 14 nonaffected family members also did not tolerize to KLH, suggesting that some of these family members might carry the genetic determinant for the loss of tolerance but did not experience IBD [52]. Either they lack exposure to an environmental factor or they lack another contributing genetic factor for the development of IBD.

These studies suggest that oral tolerance therapy for IBD would not be effective; however, in the preliminary studies to date, the results were more promising than other oral tolerance therapeutic trials. Ilan [53*] headed a study with

ENZO Biochem in Israel in which 10 Crohn's disease patients with Crohn's disease activity index (CDAI) scores between 200 and 350 were selected and underwent colonoscopies wherein colonic material underwent biopsy and the specimens were prepared for their own orally fed antigenic preparation. After a 16-week treatment of three feedings a week, a median decrease in the CDAI of 129 after 14 weeks was observed, although the score did rise again after treatment ended. Subsets of patients had a reduction of interferon- γ -secreting peripheral blood T cells specific for the fed colonic preparation, an increase of peripheral natural killer T cells, and increases in serum interleukin-4 and interleukin-10 levels. Further multicenter trials are pending from this company.

Conclusion

The gastrointestinal tract uses a profound system of tolerance and controlled inflammation to limit the response to innocuous dietary or bacteria-derived antigens in the gut. When this complex system breaks down, either by a chemical or pathogenic insult, a genetic disturbance, or both, the resulting immune response leads to colitis in mice or IBD in humans.

In a competent mucosal immune system, oral tolerance has been successfully used in laboratories to prevent or to treat mice with a variety of experimental autoimmune disorders with the hope of someday applying this therapy to human disease; however, there seem to be fundamental differences in the mechanisms underlying mouse and human oral tolerance. These differences might partially explain why experimentally induced tolerance is less effective in humans than in mouse models, and why human trials of oral tolerance therapy for inflammatory and autoimmune disorders are less successful than oral tolerance therapy in mouse models.

Using oral tolerance therapy for IBD is complicated further by the fact that the mucosal immune system is already compromised. Statistical analyses of familial inheritance patterns suggest that IBD is a multigenetic disorder. If this is true, then the same nonfunctional or partially effective mucosal immune system that led to IBD is still defective in terms of an ability to tolerize. Indeed, studies from our laboratory suggest that the same protocol that results in oral tolerance in control individuals leads instead to an activation of the immune system in IBD patients. These results suggest that oral tolerance therapy would be ineffective in IBD patients. It is possible, however, that oral tolerance responses to neoantigens are different from those to self-antigens, commensal bacterially derived antigens, or both. Alternatively, the dose and regimen of feeding could lead to differing results.

With a greater understanding of the mechanisms governing the responses of the human mucosal immune

system and oral tolerance will come greater hope for effective treatment of IBD and other inflammatory and autoimmune disorders.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 738–739).

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- This is the only oral tolerance trial for IBD. The results are more favorable than those of oral tolerance trials for other diseases to date.

EXHIBIT 13

In *Trans* T Cell Tolerance Diminishes Autoantibody Responses and Exacerbates Experimental Allergic Encephalomyelitis¹

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A number of Ag-specific approaches have been developed that ameliorate experimental allergic encephalomyelitis (EAE), an animal model for the human autoimmune disease multiple sclerosis. Translation to humans, however, remains a consideration, justifying the search for more insight into the mechanism underlying restoration of self-tolerance. Ig-proteolipid protein (PLP) 1 and Ig-myelin oligodendrocyte glycoprotein (MOG) are Ig chimeras carrying the encephalitogenic PLP 139–151 and MOG 35–55 amino acid sequence, respectively. Ig-PLP1 ameliorates EAE in SJL/J (H-2^b) mice while Ig-MOG modulates the disease in C57BL/6 (H-2^b) animals. In this study, we asked whether the chimeras would suppress EAE in F₁ mice expressing both parental MHC alleles and representing a polymorphism with more relevance to human circumstances. The results show that Ig-MOG modulates both PLP1 and MOG peptide-induced EAE in the F₁ mice, whereas Ig-PLP1 counters PLP1 EAE but exacerbates MOG-induced disease. This *in trans* aggravation of MOG EAE by Ig-PLP1 operates through induction of PLP1-specific T cells producing IL-5 that sustained inhibition of MOG-specific Abs leading to exacerbation of EAE. Thus, in *trans* T cell tolerance, which should be operative in polymorphic systems, can aggravate rather than ameliorate autoimmunity. This phenomenon possibly takes place through interference with protective humoral immunity. *The Journal of Immunology*, 2008, 180: 1508–1516.

Antigen-specific therapy has become an attractive approach for the treatment of autoimmune diseases because it specifically targets the responses associated with the pathology of the disease, avoiding major side effects (1, 2). Many approaches have proven effective against experimental allergic encephalomyelitis (EAE),⁴ but a very limited number of these have efficiently translated into therapies for human multiple sclerosis (MS) and have had only partial effects (2, 3).

Delivery of Ag on Ig has proven to be promising against T cell-mediated autoimmunity including EAE and type 1 diabetes (4–7). Indeed, it has been shown that Ig-proteolipid protein 1 (PLP1), an Ig-encompassing PLP1 peptide corresponding to amino acid sequence 139–151 of PLP (8), is effective against the relapses of EAE (4). Upon aggregation, however, Ig-PLP1 was able to

cross-link FcγRs and induce the production of suppressive cytokines such as IL-10 by APCs (4, 8). Consequently, aggregated (agg) Ig-PLP1 was effective in modulating the initial severe phase of PLP1-induced disease, as well as in suppressing the relapses (4). Additionally, agg Ig-PLP1 was able to reverse EAE induced in SJL/J mice by a CNS homogenate, which contains multiple encephalitogenic determinants and provides a full spectrum of pathological responses (5). The approach was not restricted to Ig-PLP1 because agg Ig-myelin oligodendrocyte glycoprotein (MOG), an Ig-encompassing MOG 35–55 peptide, was also able to induce IL-10 production by APCs, display bystander suppression, and reverse CNS homogenate-induced EAE in C57BL/6 mice (5). These observations suggest that the Ig delivery approach may have potential for use under circumstances involving complex MHC polymorphism and diverse T cell specificities. To test these premises, we sought to examine the therapeutic efficacy of agg Ig-PLP1 and Ig-MOG in a setting with more relevance for human MS. Accordingly, Ig-PLP1 and Ig-MOG were used to treat EAE in F₁ mice bred from crossing SJL/J and C57BL/6 mice, two genetically distinct parental strains in which the chimeras were able to reverse the disease. Because these F₁ mice would have a more complex MHC haplotype than their parents, they might give clues to define regimens that would be beneficial for therapy of human autoimmunity. Here, it is shown that both Ig-PLP1 and Ig-MOG are effective against CNS homogenate-induced EAE in (SJL × B10.PL)F₁ mice. However, (SJL × C57BL/6)F₁ animals were resistant to treatment with either chimera. Interestingly, when Ig-MOG was used to treat peptide-induced EAE in the (SJL × C57BL/6)F₁ mice, it showed efficacy against both PLP1- and MOG-induced EAE. However, Ig-PLP1 was effective at suppressing PLP1-induced disease but exacerbated MOG-induced EAE. Evidence is provided indicating that exacerbation of disease by Ig-PLP1 is sustained by PLP1-reactive lymphocytes. Indeed, upon induction of EAE by MOG peptide and treatment with Ig-PLP1, T cells specific

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⁴ Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis; PLP, proteolipid protein; agg, aggregated; MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein; HA, hemagglutinin; SP, spleen; LN, lymph node.

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for PLP1 peptide produced IL-5 cytokine which inhibited the production of MOG-specific Abs leading to exacerbation rather than amelioration of EAE. Thus, T cell tolerance that sustains amelioration of EAE in inbred mice evolved to negate protective humoral immunity in the more polymorphic F_1 mice and aggravates autoimmunity.

Materials and Methods

Animals

SJL/J (H-2^b), C57BL/6 (H-2^b), and B10.PL (H-2^b) mice were purchased from The Jackson Laboratory. F_1 (SJL/J \times C57BL/6) and (SJL/J \times B10.PL) mice were generated by breeding male SJL/J to female C57BL/6 and B10.PL, respectively. All mice were maintained in our animal care facility for the duration of the experiments. All experimental procedures were performed according to the guidelines of the institutional animal care committee.

Antigens

Peptides. The peptides used in this study were purchased from Metabion and were HPLC purified to >90% purity. PLP1 peptide (HSLGKWLGH PDKF) encompasses amino acid residues 139–151 of PLP and is encephalitogenic in SJL/J mice (9). MOG peptide (MEVGWYRSPFSRVVH LYRNGK), encompassing amino acid residues 35–55 of MOG, is encephalitogenic in C57BL/6 and B10.PL mice (10). PLP2 peptide (NT WTTCQSIAPFSK) comprising amino acid residues 178–191 of PLP is also encephalitogenic in the SJL/J mouse (11). Myelin basic protein 3 (MBP3) peptide (VHFFKNIVTPRTTP) corresponds to amino acid residues 87–99 of MBP and is encephalitogenic in the SJL/J mouse (12). PLP-LR (HSLGKLLGRPDKF) is a mutant form of PLP1 in which Trp¹⁴⁴ and His¹⁴⁷ were replaced with Leu and Arg, respectively, and serves as an antagonist to PLP1 (13). Influenza virus hemagglutinin (HA) amino acid residues 110–120 peptide (SFERFEIFPKI) was used as a negative control (4).

CNS homogenate. Fifty frozen unstripped rat brains (Pelfreez Biologicals) were homogenized in PBS using a Waring blender and adjusted to 300 mg/ml with PBS (4, 5).

Ig chimeras. Ig-PLP1 chimera, harboring PLP1 peptide within the H chain CDR3, has been shown to be effective against EAE when injected into mice in saline (14). Similarly, Ig-MOG harboring MOG 35–55 peptide (5), Ig-MBP3 carrying MBP87–99 (15), and Ig-PLP2 encompassing PLP178–191 (16), have been shown to function as tolerogens when given to mice without adjuvant (4, 5, 15, 16). Ig-PLP-LR, which incorporates PLP-LR altered peptide, functions as an antagonist for PLP1-specific T cells (14). All Ig chimeras have, like Ig-PLP1, had the peptide of interest inserted within the H chain CDR3 region and were constructed using the genes coding for the IgG2b, κ anti-arsonate Ab 91A3 as described previously (14). In brief, the D segment was deleted from the CDR3 of the H chain V region and replaced with a nucleotide sequence that codes for the peptide using mutagenesis procedures similar to those described for the generation of Ig-PLP1 (14). The resulting 91A3-peptide chimeric IgG2b H chain was cotransfected with the parental 91A3 κ chain into the non-Ig-producing SP2/0 myeloma B cell line, and the transfectoma cells producing complete Ig-PLP1 were selected with drugs as described previously (14). Transfection, cloning, sequencing, and purification procedures for Ig-MOG, Ig-MBP3, Ig-PLP2, and Ig-PLP-LR are similar to those used for Ig-PLP1 (5–8, 14).

Aggregation of the Ig chimeras

The chimeras were aggregated by precipitation with 50%-saturated $(\text{NH}_4)_2\text{SO}_4$ as has been previously described (4). Because all chimeras are derived from the same Ig backbone and thereby comprise identical IgG2b isotypes, their Fe-associated functions will be similar.

Induction and scoring of EAE

Induction of EAE has been described previously (4, 5). Briefly, mice (6–8 wk old) were induced for EAE by s.c. injection in the footpads and at the base of the limbs with a 200- μ l IPA/PBS (v/v) solution containing 6 mg of CNS homogenate, 100 μ g of PLP1, or 300 μ g of MOG, along with 200 μ g of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories). Six hours later, the mice were given i.v. either 200 (SJL/J \times B10.PL) or 500 (SJL/J \times C57BL/6) ng of purified *Bordetella pertussis* toxin (List Biological Laboratories). A second injection of *B. pertussis* toxin was given after 48 h. The mice were then scored daily for clinical signs of EAE as follows: 0, no clinical score; 1, loss of tail tone; 2, hind

limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; and 5, moribund or death.

Treatment of EAE

Standard treatment regimen. Mice were treated three times, 4 days apart with 300 μ g of agg Ig chimeras at the first observation of clinical signs as described previously (4, 5). Typically, the treatments were given on days 13, 17, 21 postdisease induction by i.p. injection unless indicated otherwise.

Extended treatment regimen. In some experiments the F_1 (SJL/J \times C57BL/6) mice received a prolonged treatment regimen consisting of 300 μ g of agg Ig chimeras at the first observation of clinical signs and then every 4 days for the duration of the observation period.

In vivo antagonism of PLP1-specific T cells

In some experiments, antagonism of PLP1-specific T cells was conducted during treatment with agg Ig-PLP1. In this case, the mice were induced for EAE with MOG peptide and treated three times, 4 days apart with a mixture of 300 μ g of agg Ig-PLP1 and 300 μ g of agg Ig-PLP-LR at the first observation of clinical signs as described above.

In other experiments, antagonism of PLP1-specific T cells was conducted before induction of EAE or treatment with agg Ig-PLP1. Accordingly, the mice were given soluble (nonaggregated) Ig-PLP-LR on days -10, -6, and -3 before induction of EAE with MOG peptide. Soluble Ig-PLP-LR was used instead of agg Ig-PLP-LR because this form has been shown to antagonize PLP1-specific T cells (8, 14) and does not induce the production of IL-10 by APCs which could down-regulate unrelated T cells by bystander suppression. When the signs of EAE were apparent, the mice were treated with agg Ig-PLP1 according to the standard regimen.

Detection of IFN- γ and IL-5 by ELISA

ELISA was performed according to BD Pharmingen standard protocol. The capture Abs were as follows: rat anti-mouse IFN- γ , R4-6A2 and rat anti-mouse IL-5, TRFK-5. The biotinylated anti-cytokine Abs were rat anti-mouse IFN- γ , XMGI.2, and rat anti-mouse IL-5, TRFK-4. The OD₄₀₅ was read on a SpectraMAX 190 counter (Molecular Devices) and analyzed using SOFTmax PRO 3.1.1 software. Graded amounts of recombinant mouse IFN- γ and IL-5 (BD Pharmingen) were included for construction of a standard curve. The cytokine concentration in culture supernatants was extrapolated from the linear portion of the standard curve.

Inhibition of IFN- γ production by anti-CD4 Ab

F_1 (SJL/J \times C57BL/6) mice were induced for EAE with MOG peptide and treated with agg Ig-PLP1 according to the standard treatment regimen. The spleen (SP) and lymph node (LN) anti-MOG IFN- γ responses were then analyzed 3 days after the final treatment. Accordingly, SP or LN cells (1×10^6 cells/well/100 μ l) were stimulated with 30 μ g/ml MOG peptide (50 μ l/well) and 20 μ g/ml (50 μ l/well) of anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), or anti-H-2D^b (clone 28-14-8) Ab. For the combination of anti-CD8 plus anti-H-2D^b, 20 μ g/ml of each was added. Rat IgG and mouse IgG were included as controls for Abs in matching doses. Cell cultures were incubated for 24 h at 37°C, after which culture supernatants were transferred to anti-IFN- γ -coated plates and cytokine was detected by ELISA as indicated above.

Detection of MOG-specific Abs by ELISA

For detection of anti-MOG Abs in the serum of MOG/EAE mice treated with agg Ig-PLP1, ELISA was used according to the following protocol. Fifty microliters of 0.1 M bicarbonate buffer containing 7 μ g/ml MOG peptide was coated into 96-well plates and incubated overnight at 4°C. The plates were then washed and saturated with PBS-3% BSA. Subsequently, serial dilutions of serum were added and incubated overnight at 4°C. Total anti-MOG Abs were detected using anti- κ and anti-mouse Ig coupled to HRP from the Southern Biotechnology Associates clonotyping kit according to the manufacturer's instructions. Class and subclass isotyping were performed in a similar manner using anti-IgG1, -IgG2a and 2b, -IgG3, -IgM, -IgA obtained from the Southern Biotechnology Associates clonotyping kit.

Statistical analysis

Statistical significance comparing EAE disease curves was analyzed by two-way mixed model ANOVA test using GraphPad Prism software

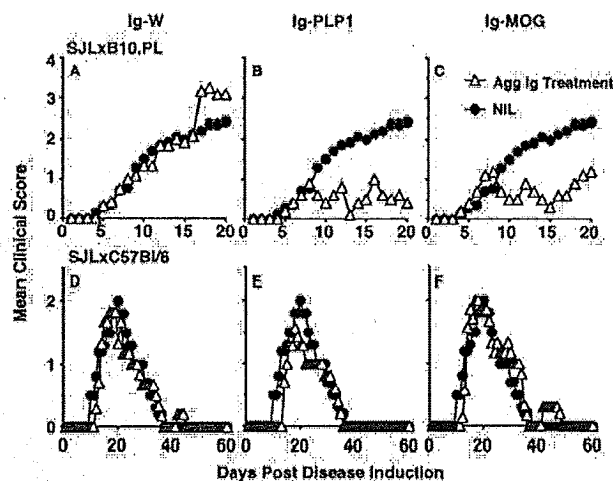


FIGURE 1. Treatment of CNS homogenate-induced EAE in genetically distinct F_1 mice with Ig chimeras shows differential efficacy. Groups of 6- to 8-wk-old (SJL \times B10.PL) (A-C) or (SJL \times C57BL/6) (D-F) F_1 mice were induced for EAE by s.c. injections of 6 mg of CNS homogenate along with 200 μ g of H37Ra in a 200- μ l suspension of PBS/IFA (v/v). When the clinical signs of EAE became evident (loss of tail tone), the mice were then treated i.p. with 300 μ g of agg Ig-PLP1 (B and E), agg Ig-MOG (C and F), or control agg Ig-W (A and D) three times at 4-day intervals. The animals were then scored daily for the indicated periods of time.

(GraphPad Software). Significance is indicated as p values in corresponding figure legends with p values <0.05 considered significant and <0.01 highly significant.

Results

Ig-myelin chimeras reverse EAE in (SJL/J \times B10.PL) but not (SJL/J \times C57BL/6) F_1 mice

Reports indicated that agg Ig-PLP1 (14) and Ig-MOG (5) can reverse CNS homogenate-induced EAE involving diverse T cell specificities (4, 5). The mechanism underlying such effectiveness likely involves cytokine-mediated bystander suppression along with minimal costimulation (4, 5, 8). Because the previous studies were conducted in inbred animals with restricted MHC haplotypes, we sought to test the Ig-myelin system in a more polymorphic animal model, which would be more relevant to human circumstances. As such, we generated two types of F_1 mice, an (SJL/J \times B10.PL) and an (SJL/J \times C57BL/6) F_1 strain, and tested the agg Ig-myelin chimeras for suppression of EAE. Accordingly, the F_1 female mice were induced for EAE with CNS homogenate, and when the clinical signs of disease became apparent, the animals were given three injections of 300 μ g of agg Ig-PLP1 or agg Ig-MOG in saline at 4-day intervals and assessed for reduction in disease severity. Control mice were given agg Ig-W, the parental Ig backbone without any peptide insert. Fig. 1 shows that in the (SJL/J \times B10.PL) F_1 mice, those treated with agg Ig-W, similar to untreated animals, had a severe chronic form of EAE, while both agg Ig-PLP1- and agg Ig-MOG-treated animals had mild clinical signs of EAE (Fig. 1, upper panels). In contrast, in the (SJL/J \times C57BL/6) F_1 mice, while the CNS homogenate injection induced a milder monophasic form of EAE, the treatment with either agg Ig-PLP1, or agg Ig-MOG, had no significant effect in modulating disease (Fig. 1, lower panels). These results indicate that the ability of agg Ig-myelin chimeras to treat CNS homogenate-induced EAE depends on the genetic make up of the F_1 mice and, probably, the resulting dominant epitopes.

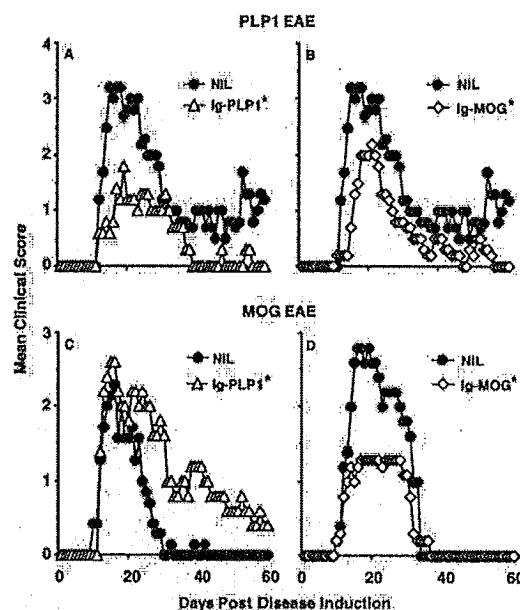


FIGURE 2. Agg Ig-PLP1 treatment exacerbates MOG peptide-induced EAE in SJL \times C57BL/6 F_1 mice. Groups (six to eight mice per group) of 6- to 8-wk-old (SJL \times C57BL/6) F_1 mice were induced for EAE by s.c. injections of 200 μ l of PBS/IFA (v/v) containing 200 μ g of H37Ra and either (A and B) 100 μ g of PLP1 peptide or (C and D) 300 μ g of MOG peptide. The mice were treated three times every 4 days with 300 μ g of either agg Ig-PLP1 (A and C) or agg Ig-MOG (B and D) beginning at the first observation of clinical symptoms (day 13) and scored daily for the indicated periods of time. Groups of mice were left untreated (NIL) for comparison purposes. *, Values of p < 0.05 as determined by two-way ANOVA described in Materials and Methods.

F_1 (SJL/J \times C57BL/6) mice induced for EAE with MOG peptide exacerbate their disease when treated with agg Ig-PLP1 but not agg Ig-MOG

Because both agg Ig treatments were able to suppress CNS homogenate-induced EAE in the (SJL/J \times B10.PL) but not in the (SJL/J \times C57BL/6) F_1 mice, we sought to determine whether similar effects would occur when the disease is induced by a single, rather than multiple, determinant(s). Accordingly, (SJL/J \times C57BL/6) F_1 mice were induced for EAE with either PLP1 (SJL/J-restricted) or MOG (C57BL/6-restricted) peptide and each disease (designated PLP1/EAE and MOG/EAE, respectively) was treated with either agg Ig-PLP1 or agg Ig-MOG. As can be seen in Fig. 2, animals with ongoing PLP1/EAE reduced the severity of their disease when treated with either agg Ig-PLP1 or agg Ig-MOG (upper panels). In fact, untreated mice with ongoing PLP1/EAE showed a mean maximal disease severity score of 3.2 ± 0.8 and did not recover during the 60-day monitoring period (Table I). However, mice which were treated with agg Ig-PLP1 recovered by day 37 postdisease induction and exhibited a mean maximal clinical severity of 1.8 ± 0.4 . Also, the agg Ig-MOG-treated animals recovered by day 33 with a mean maximal disease severity score of 2.2 ± 0.8 . In contrast, mice induced for EAE with MOG peptide showed a significant reduction in disease severity when treated with agg Ig-MOG, but those treated with agg Ig-PLP1 had a pronounced exacerbation of the disease relative to untreated control animals (lower panels). The untreated animals with ongoing MOG/EAE had a mean maximal severity of 2.3 ± 0.5 and recovered by day 27. When the disease was treated with agg Ig-MOG, the animals showed a mean maximal disease severity of 1.3 ± 0.5 .

Table I. Treatment with agg Ig-PLP1 exacerbates MOG-induced EAE^a

Disease Inducer	Treatment with	Day of Disease Onset ^b	Mean Maximal Disease Severity	Day of Recovery ^c
PLP1	Nil	12.0 ± 0.0	3.2 ± 0.8	>60
	agg Ig-PLP1	13.2 ± 2.2	1.8 ± 0.4	37
	agg Ig-MOG	15.3 ± 2.6	2.2 ± 0.8	33
MOG	Nil	11.1 ± 1.0	2.3 ± 0.5	27
	agg Ig-PLP1	12.4 ± 0.5	2.6 ± 0.5	>60
	agg Ig-MOG	11.7 ± 1.0	1.3 ± 0.5	32

^aThe data illustrated in this table were obtained from the mice described in the legend to Fig. 2. These animals were induced for EAE with either PLP1 or MOG peptide and treated with agg Ig-PLP1 or agg Ig-MOG according to the standard regimen.

^bThe onset of EAE represents the day a mouse receives a score of 1 or above, and the indicated numbers represent the mean ± SD.

^cMice were considered recovered when their mean clinical score was <0.5 for >3 consecutive days.

and recovered by day 32. To the contrary, mice which were treated with agg Ig-PLP1 showed exacerbated MOG/EAE with a mean disease score of 2.6 ± 0.5 and the mice did not recover for the entire 60-day monitoring period. These results indicate that agg Ig-MOG is effective at treating both PLP1- and MOG-induced EAE, but that agg Ig-PLP1, while effective at suppressing PLP1/EAE, exacerbates MOG-induced EAE and significantly delays spontaneous recovery.

To ensure that exacerbation of disease is not due to spreading to PLP1 epitope after completion of the treatment, the regimen was extended for a longer period of time and the animals were monitored for reversal of disease. As can be seen in Fig. 3, when compared with a group that was given only the standard three-injection regimen of agg Ig-PLP1, the mice that received the extended treatment regimen showed no reduction in disease severity. In fact, the mean maximal disease severity score was 2.4 ± 0.5 which is similar to the 2.5 ± 0.6 score of mice recipient of the standard regimen. Furthermore, both treatment regimens exacerbated the disease and resolution was delayed significantly. Indeed, whereas the untreated (NIL) group showed recovery by day 30 postdisease in-

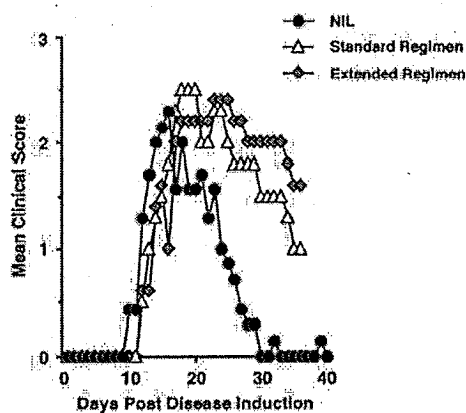


FIGURE 3. Exacerbation of MOG-induced EAE persists even with a continuous agg Ig-PLP1 treatment regimen. Groups (six to eight mice per group) of 6- to 8-wk-old (SJL × C57BL/6)F₁ mice were given 200 μl of PBS/IFA (v/v) s.c. injections containing 300 μg of MOG peptide and 200 μg of H37Ra. At the onset of disease (loss of tail tone), a group of mice was given agg Ig-PLP1 three times at 4 days interval (standard treatment regimen), another group was given agg Ig-PLP1 every 4 days for the duration of the experiment (extended treatment regimen) and a third group was left untreated (NIL) to serve as control. The mice were then scored daily for the indicated period of time.

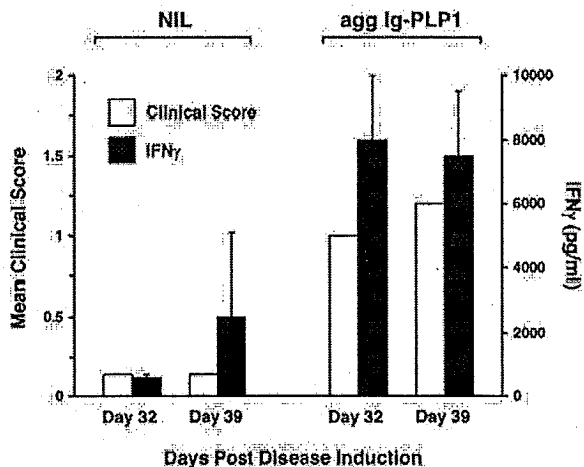


FIGURE 4. agg Ig-PLP1 treatment prolongs MOG-specific IFN-γ production. Groups of 6- to 8-wk-old (SJL × C57BL/6)F₁ mice were induced for EAE by s.c. injection of 200 μl of PBS/IFA (v/v) containing 300 μg of MOG peptide and 200 μg of H37Ra. A group of mice was treated three times every 4 days with agg Ig-PLP1 (agg Ig-PLP1) beginning on the day when clinical signs of disease were first observed. An untreated group (NIL) was included for control purposes. The mice were then sacrificed at day 32 or 39 postdisease induction and their LN IFN-γ responses to MOG peptide were analyzed by ELISA. Each filled bar (■) represents the mean ± SD IFN-γ production from six to eight mice. Each open bar (□) represents the mean clinical score of the corresponding group of mice.

duction, those treated with the standard or extended agg Ig-PLP1 treatment regimen did not recover by completion of the monitoring period and had a residual mean disease score of 1.5 ± 0.6 and 2.0 ± 0.0 , respectively. These results suggest that exacerbation of EAE upon treatment with agg Ig-PLP1 is not due to spreading to PLP1-specific T cells after completion of the short treatment regimen.

agg Ig-PLP1 treatment of MOG-induced EAE sustains IFN-γ production from MOG-reactive cells

To investigate the effects of agg Ig-PLP1 treatment on the MOG-specific T cells, we chose to examine Ag-specific IFN-γ production throughout the course of MOG/EAE with and without agg Ig-PLP1 treatment. Accordingly, (SJL × C57BL/6)F₁ mice were induced for EAE with MOG peptide and treated with agg Ig-PLP1 with the standard (three injections) treatment regimen. On day 32, a point at which the disease resolves in the untreated mice but remains severe in the treated animals, the MOG T cell response was analyzed by measuring MOG-specific IFN-γ production. Interestingly, as shown in Fig. 4, IFN-γ production mirrored the clinical severity of disease in both the treated and untreated mice. Indeed, MOG-specific IFN-γ is clearly produced at higher levels in the LN of agg Ig-PLP1-treated mice as compared with the untreated animals. This is at a time during the disease course in which the treated mice are unable to resolve their disease, and the mean clinical score is significantly higher than in the NIL group. Similar results were observed on day 39 where disease resolution in the untreated mice is maintained, while signs of paralysis remain significant in the treated animals (Fig. 4). Again, IFN-γ production parallels with disease status and was minimal for the NIL group but highly significant in the treated mice. Therefore, it appears that MOG-specific T cells remain active during exacerbation of disease during treatment with agg Ig-PLP1.

Administration of agg Ig-PLP1 has been previously shown to suppress EAE at least partially through the induction of IL-10 production by APCs (4, 5). Because IL-10 can serve as a growth

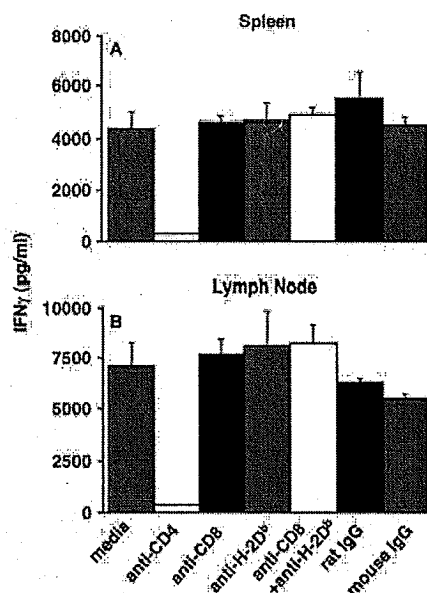


FIGURE 5. MOG-specific CD4, not CD8, T cells mediate the anti-MOG response upon agg Ig-PLP1 treatment of MOG-induced EAE. Groups of 6- to 8-wk-old (SJL × C57BL/6)F₁ mice were induced for EAE with MOG peptide as in Fig. 3. The mice were treated three times every 4 days beginning at the onset of clinical signs with 300 μg of agg Ig-PLP1. An untreated control group was included for comparison. Three days after the final treatment, the mice were sacrificed and their (A) SP and (B) draining LN cells were stimulated with MOG peptide in the presence or absence of blocking anti-CD4, -CD8, or -H-2D^b Abs. The SP and LN cells were used at 1×10^6 cells/100 μl/well. The stimulation used 30 μg/ml MOG peptide and 20 μg/ml anti-CD4, -CD8, or -H-2D^b (anti-class I) Ab. Combination of Abs uses 20 μg/ml each. Rat and mouse IgG were included as controls for Abs in matching doses. Each bar represents the mean ± SD of triplicate wells. The results are representative of two independent experiments.

factor for CD8 T cells (17) and it was recently reported that MOG 35–55 encompasses a CD8 epitope (18, 19), we wanted to test whether the IL-10 produced by the APCs upon agg Ig-PLP1 administration was serving to help expand MOG-specific CD8 T cells to exacerbate the disease. To this end, (SJL × C57BL/6)F₁ mice were induced for EAE with MOG peptide and treated three times with agg Ig-PLP1 according to the standard treatment regimen beginning at the first clinical signs of disease. Three days following the final treatment (day 24), the mice were sacrificed and their SP and LN IFN-γ responses to MOG peptide stimulation in the presence of anti-CD4, -CD8, or -H-2D^b Abs were measured by ELISA. As can be seen in Fig. 5, and like the results shown in Fig. 4, there is still a significant IFN-γ production in both the SP and LN of the treated mice (medium). At this time point, the response is comparable to that from mice that had received no treatment when examined at the same time point (data not shown), indicating that agg Ig-PLP1 administration, although able to prolong the MOG response, does not increase the magnitude of the MOG-specific T cell response. When anti-CD4 Ab was added to the cultures, the responses in both the SP (*upper panel*) and LN (*lower panel*) were significantly reduced, indicating that CD4 T cells are responsible, at least in part, for mediating MOG responses. When anti-CD8 Ab or anti-class I (H-2D^b) Ab were added, no change was seen when compared with that of the normal response (medium). Taken together, these results indicate that treatment with agg Ig-PLP1 sustains activation of MOG-specific CD4, not CD8, T cells.

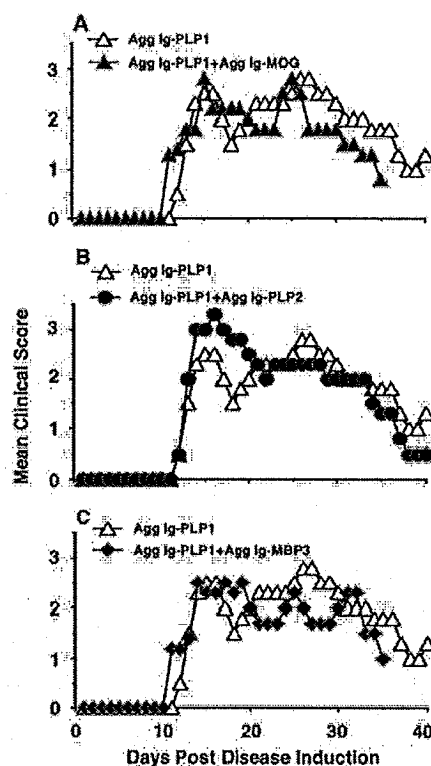


FIGURE 6. agg Ig-PLP1 treatment continues to exacerbate MOG-induced EAE when administered with Igs carrying other myelin epitopes. Groups of 6- to 8-wk-old (SJL × C57BL/6)F₁ mice were induced for EAE with 300 μg of MOG peptide as in Fig. 3. At the onset of clinical signs (day 13), the mice were given injections of (A) agg Ig-PLP1 + agg Ig-MOG, (B) agg Ig-PLP1 + agg Ig-PLP2, or (C) agg Ig-PLP1 + agg Ig-MBP3. Each mixture contained 300 μg of agg Ig-PLP1 and 300 μg of the additional chimera. A group of mice treated with 300 μg of agg Ig-PLP1 alone was included for control purposes.

Spreading to epitopes other than PLP1 and MOG is not responsible for exacerbation of MOG-induced EAE by treatment with agg Ig-PLP1

Exacerbation of EAE by agg Ig-PLP1 may have resulted from activation of unrelated T cells as a consequence of a pattern of epitope spreading (20–22) dictated by the F₁ MHC polymorphism. To address this issue, we sought to induce EAE with MOG peptide, treat with agg Ig-PLP1 along with other chimeras carrying different epitopes, and monitor for reduction in disease severity. Three treatment regimens were tested which include agg Ig-PLP1 in combination with agg Ig-MOG; agg Ig-PLP1 along with agg Ig-PLP2 (16), a chimera carrying the I-A^b-restricted PLP2 peptide corresponding to amino acid residues 179–191 of PLP; and agg Ig-PLP1 combined with agg Ig-MBP3 (15) a chimera carrying the promiscuous amino acid sequence 87–99 of MBP. These regimens were applied in the form of a mixture of equal amounts of chimera (300 μg each) according to the standard three injections at 4-day intervals. A group of mice recipient of agg Ig-PLP1 alone was included to serve as a control. As can be seen in Fig. 6, none of the combinations were able to induce reduction in the severity or duration of the disease. Indeed, the mean maximal disease score was 2.8 ± 0.8 for treatment with agg Ig-PLP1 plus agg Ig-MOG; 3.3 ± 1.0 for agg Ig-PLP1 plus agg Ig-PLP2; and 2.5 ± 1.0 for the treatment with agg Ig-PLP1 plus agg Ig-MBP3. This is similar to the 2.8 ± 1.0 score observed during treatment with agg Ig-PLP1

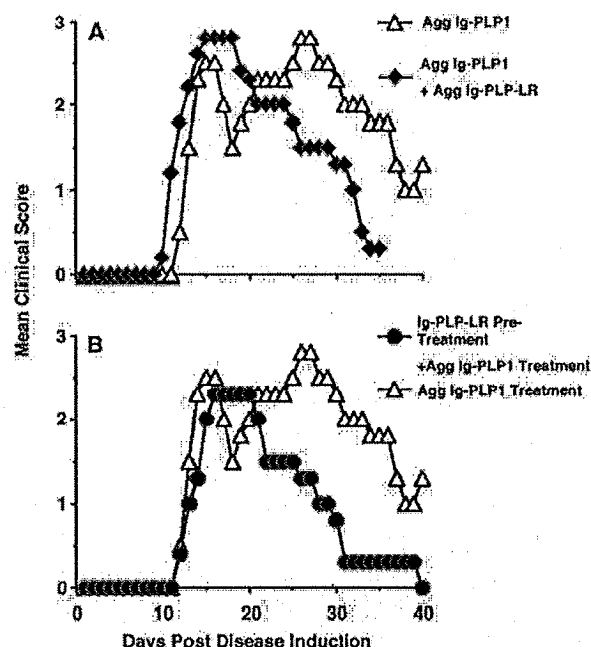


FIGURE 7. Antagonism of PLP1-specific T cells by Ig-PLP-LR nullifies agg Ig-PLP1-mediated exacerbation of MOG-induced EAE. *A*, Groups of 6- to 8-wk-old (SJL \times C57BL/6) F_1 mice were induced for EAE with 300 μ g of MOG peptide and treated three times every 4 days beginning at the onset of clinical signs with either 300 μ g of agg Ig-PLP1 alone or in combination with 300 μ g of agg Ig-PLP-LR antagonist. The mice were monitored daily and assessed for clinical scores throughout the observation period. *B*, Five- to 7-wk-old (SJL \times C57BL/6) F_1 mice were given 300 μ g of soluble Ig-PLP-LR i.p. at days -10, -6, and -3 before disease induction. These mice along with a group of 6- to 8-wk-old untreated (SJL \times C57BL/6) F_1 mice were then induced for EAE with 300 μ g of MOG peptide. At the onset of clinical symptoms, both groups of mice were treated three times, 4 days apart with 300 μ g of agg Ig-PLP1 and monitored for clinical signs of EAE daily until day 40 post disease induction.

alone. Moreover, the patterns of clinical signs were similar throughout the duration of the monitoring time period. Thus, the exacerbation of MOG/EAE by agg Ig-PLP1 is not due to spreading to other epitopes such as PLP2 or MBP3. Interestingly, even agg Ig-MOG when combined with agg Ig-PLP1 is no longer able to modulate the disease.

Antagonism of PLP1-specific cells by Ig-PLP-LR modulates agg Ig-PLP1-mediated disease exacerbation

Epitope spreading does not seem to be responsible for exacerbation of MOG-induced EAE by agg Ig-PLP1. Treatment with Ig-MOG combined with Ig-PLP1 does not modulate the disease possibly indicating that MOG-specific T cells may not be responsible for exacerbation of disease. Thus, it may be that agg Ig-PLP1 in the context of H-2^b stimulates rather than inactivates PLP1-specific T cells to sustain the severity of EAE. To test this premise, we sought to antagonize PLP1-specific T cells and determine whether disease exacerbation by agg Ig-PLP1 persists.

It has previously been shown that replacement of the TCR contact residues 144W and 147H with 144L and 147R of PLP1 generates a peptide designated PLP-LR that functions as an antagonist of PLP1-specific T cells (13). Also, PLP-LR was previously expressed on Ig and the resulting Ig-PLP-LR chimera antagonized PLP1-specific T cells effectively (8, 14). Herein, Ig-PLP-LR was used to antagonize PLP1-specific T cells to test for the effect of agg

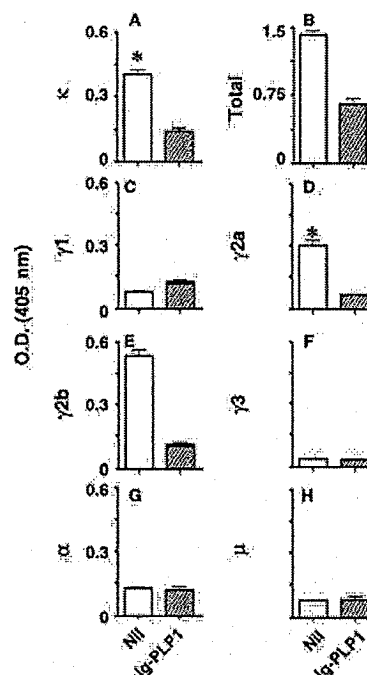


FIGURE 8. agg Ig-PLP1 treatment diminishes anti-MOG Abs. Groups of 6- to 8-wk-old (SJL \times C57BL/6) F_1 mice were induced for EAE with 300 μ g of MOG peptide and treated with 300 μ g of agg Ig-PLP1 three times at 4-day intervals beginning at the onset (loss of tail tone) of clinical signs of EAE. The mice were bled via tail veins 4 days after the last agg Ig-PLP1 injection. A group of mice induced for MOG/EAE but treated with PBS instead of agg Ig-PLP1 was included for comparison purposes. κ -bearing (*A*) and total (*B*) MOG Abs from the untreated (Nil) and agg Ig-PLP1-treated (Ig-PLP1) mice were measured by ELISA using the Southern Biotechnology Associates kit as described in *Materials and Methods*. Isotyping for γ 1 (*C*), γ 2a (*D*), γ 2b (*E*), γ 3 (*F*), α (*G*), and μ (*H*) class and subclass of anti-MOG Ab were also determined by ELISA using the Southern Biotechnology Associates kit as described in *Materials and Methods*. Serial serum dilutions were used in these analyses and the results shown were those obtained with 1/500 dilution. Each bar represents the mean \pm SD of triplicates from five mice. *, Significant difference relative to control untreated mice as analyzed by Student *t* test.

Ig-PLP1 on MOG-induced EAE. Accordingly, (SJL \times C57BL/6) F_1 mice were induced for EAE with MOG peptide and when the clinical signs of disease became apparent, the mice were given agg Ig-PLP1 alone or in combination with agg Ig-PLP-LR and monitored daily for reduction in disease severity. As illustrated in Fig. 7*A*, while the mean maximal score remains unchanged in the two groups (2.8 ± 0.8 vs 2.8 ± 1.0), the mice treated with the agg Ig-PLP1 and agg Ig-PLP-LR combination regimen reduced the severity of their paralysis earlier and resolved their disease by day 35 following disease induction. To ensure that exacerbation of disease involves PLP1-specific T cells, we used soluble Ig-PLP-LR to antagonize the cells before disease induction with MOG and treated with agg Ig-PLP1 alone. The choice of soluble Ig-PLP-LR for T cell antagonism before disease induction stems from the observation that this form is effective for prevention of disease and circumvents IL-10 bystander suppression as soluble chimeras do not induce IL-10 production by APCs (4). As indicated in Fig. 7*B* when Ig-PLP-LR was given before disease induction the mean clinical score was slightly lower relative to mice not given Ig-PLP-LR (2.8 ± 0.5 vs 2.3 ± 0.5). However, the severity

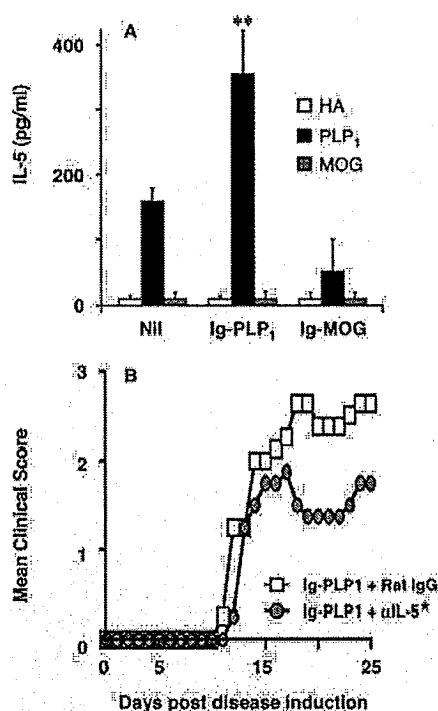


FIGURE 9. agg Ig-PLP₁ treatment exacerbates MOG/EAE through induction of IL-5 production by PLP₁-specific T cells. In A, groups of 6- to 8-wk-old (SJL × C57BL/6)_{F1} mice were induced for EAE with 300 μg of MOG peptide and treated with 300 μg of either agg Ig-PLP₁ or agg Ig-MOG three times at 4-day intervals beginning at the onset (loss of tail tone) of clinical signs of EAE. A group of mice that was induced for MOG/EAE but was not treated with any chimera (Nil) was included as control. The mice were sacrificed 4 days after the last treatment with the chimeras and their LN cells (5×10^5 cells/well) were stimulated in vitro with PLP₁ (15 μg/ml), MOG (30 μg/ml), or HA (30 μg/ml) and cytokine production was measured by ELISA as described in *Materials and Methods*. Each bar represents the mean \pm SD of triplicate wells. In B, groups of MOG/EAE mice were treated with agg Ig-PLP₁ as in A and received an injection of 500 μg of anti-IL-5 Ab (TRFK-5) or rat IgG control along with the second agg Ig-PLP₁ treatment. The mice were then monitored daily for clinical signs of EAE. Each point represents the mean score of eight mice. *, Values of $p < 0.05$ as determined by two-way ANOVA described in *Materials and Methods*.

of clinical signs declined significantly earlier and the disease resolved by day 30 while those not pretreated with Ig-PLP-LR did not recover for the 40-day disease monitoring period. Altogether, these results indicate that PLP₁-specific T cells need to be functional to contribute to disease exacerbation upon treatment of MOG-induced EAE with agg Ig-PLP₁.

Treatment of MOG EAE with agg Ig-PLP₁ induces IL-5 that inhibits anti-MOG Ab response and sustains exacerbation of disease in F₁ mice

The results presented above indicate that MOG-specific T cells are activated during treatment with agg Ig-PLP₁ but not required for exacerbation of disease while PLP₁-specific T cells are. The question then is how PLP₁-specific T cells contribute to exacerbation of disease.

MOG EAE usually involves Abs that contribute to the pathology of EAE (23, 24). If treatment with agg Ig-PLP₁ leads to an increase in the production of MOG-specific Abs, it would result in exacerbation of disease. To address this postulate, F₁ mice were immunized with MOG peptide, treated with agg Ig-PLP₁ and their

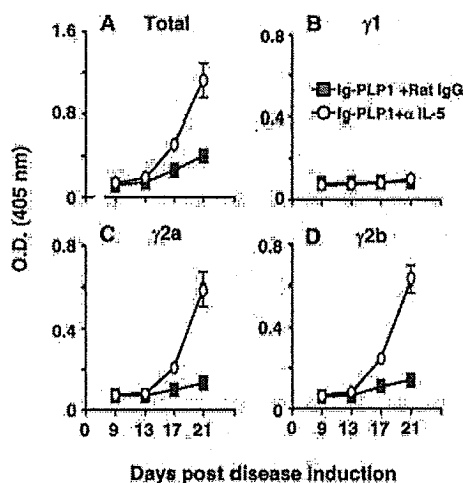


FIGURE 10. Neutralization of IL-5 during treatment of MOG/EAE with agg Ig-PLP₁ restores production of MOG-specific Abs. Groups of 6- to 8-wk-old (SJL × C57BL/6)_{F1} mice were induced for EAE with 300 μg of MOG peptide and treated with 300 μg of agg Ig-PLP₁ three times at 4-day intervals beginning at the onset (loss of tail tone) of clinical signs of EAE. An injection of 500 μg of anti-IL-5 Ab (TRFK-5) (circles) or Rat IgG control (rectangles) was also given along with the second agg Ig-PLP₁ treatment (day 13). The mice were bled via tail veins before the treatment with agg Ig-PLP₁ (day 9) and once every 4 days thereafter. Total (A) and γ1 (B), γ2a (C), and γ2b (D) isotypes of anti-MOG Abs were determined by ELISA as described in Fig. 8. Serial serum dilutions were used in these analyses and the results shown were those obtained with 1/500 dilution. Each point represents the mean \pm SD of triplicates from five mice.

sera were tested for the presence of anti-MOG Abs. The results illustrated in Fig. 8 show, surprisingly, a significant reduction rather than increase in κ bearing (Fig. 8A), as well as total, anti-MOG Abs (Fig. 8B). Furthermore, the inhibition is more prevalent for both IgG2a and IgG2b isotypes (Fig. 8, D and E) rather than IgG1, IgG3, IgA, IgM classes of Ig (Fig. 8, C and F-H).

Because PLP₁-specific T cells seem to be involved in agg Ig-PLP₁-mediated exacerbation of MOG EAE and cytokines can control the magnitude and class of Ab responses, we sought to determine whether PLP₁-specific cells and their cytokines interfere with the production of anti-MOG Abs. In an initial experiment, we immunized F₁ mice with MOG peptide, treated the animals with agg Ig-PLP₁ or control Ig-MOG that did not exacerbate the disease, and tested the T cells for differential cytokine expression. Among many cytokines tested including IFN-γ, IL-4, TGF-β, IL-17, only IL-5 was differentially produced in agg Ig-PLP₁- vs agg Ig-MOG-treated mice (Fig. 9). Indeed, IL-5 is significantly increased upon stimulation of LN cells with PLP₁ but not MOG peptide in the agg Ig-PLP₁-treated mice (Fig. 9A). However, in the mice treated with agg Ig-MOG, neither PLP₁ nor MOG peptide stimulation was able to induce significant IL-5 production. In fact, IL-5 in these mice was at background level relative to mice that were not treated with any chimeras. These results suggest that IL-5 produced by PLP₁-specific T cells may be responsible for disease exacerbation in agg Ig-PLP₁-treated MOG EAE. To test this premise, F₁ mice were induced for EAE with MOG peptide, treated with agg Ig-PLP₁ with either anti-IL-5 Ab or rat IgG control, and the severity of disease was monitored. Fig. 9B shows indeed that neutralization of IL-5 in vivo inhibits disease exacerbation and the maximal severity of paralysis was reduced from 2.3 ± 0.2 to 1.3 ± 0.4 . These results indicate that IL-5 from PLP₁-specific T cells is responsible for disease exacerbation in agg

Ig-PLP1-treated animals. Because the mice with exacerbated disease had significant IL-5, displayed diminished production of MOG Abs, and neutralization of IL-5 reduced disease severity, we suspected that IL-5 exacerbates disease by suppression of MOG Abs. If this is the case, then neutralization of IL-5 which inhibited disease severity should restore production of MOG Abs. Indeed, Fig. 10 shows that mice recipient of anti-IL-5 Ab during treatment with agg Ig-PLP1 restored production of total anti-MOG Abs (Fig. 10A). Moreover, IgG2a and IgG2b isotypes which were reduced by treatment with agg Ig-PLP1 were restored by neutralization of IL-5 (Fig. 10, C and D) but IgG1 subclass which was not affected by treatment with Ig-PLP1 was not increased by neutralization of IL-5 (Fig. 10B). These results indicate that IL-5 from PLP1-specific T cells diminishes MOG Abs and sustains exacerbation of MOG EAE in F₁ mice.

Discussion

In previous studies, we showed that both agg Ig-PLP1 and agg Ig-MOG effectively suppress EAE by a "dual modal" mechanism involving minimal costimulation and IL-10 bystander suppression (4, 5, 8). The approach is attractive because it targets both Ag-specific T cells, as well as nearby autoreactive T cells of unrelated specificities (4, 5, 8). These reports examined the efficacy of the Ig delivery system for treatment of autoimmunity in nonpolymorphic inbred mouse strains. However, these animals do not nearly represent the genetic diversity seen in humans. In this study, we examined whether the Ig approach would be able to exert similar suppressive effectiveness in a polymorphic setting with relevance to humans. Accordingly, F₁ mice were generated by breeding SJL/J to B10.PL or C57BL/6 and agg Ig-PLP1 as well as agg Ig-MOG were tested for suppression of CNS homogenate-induced EAE where the full spectrum of epitopes restricted to both parental haplotypes is at play and the chimeras would have to modulate diverse T cell specificities to ameliorate the disease. Both agg Ig-PLP1 and agg Ig-MOG were quite effective at reversing EAE induced with CNS homogenate in the (SJL/J × B10.PL)_{F1} mice (Fig. 1). Unexpectedly, however, when the same experiment was performed in the (SJL/J × C57BL/6)_{F1} strain, neither Ig chimera was able to ameliorate disease. This was intriguing and prompted investigation at the single epitope level where EAE is induced by an epitope restricted to the haplotype of one parent and the treatment is made with a chimera carrying a peptide with the same parental restriction or a peptide restricted to the other parent (in *trans* treatment). Accordingly, groups of (SJL × C57BL/6)_{F1} mice were induced for EAE with PLP1 or MOG peptide and the animals were treated with either agg Ig-PLP1 or agg Ig-MOG. It was observed that both chimeras were able to ameliorate PLP1-induced EAE (Fig. 2 and Table I). However, in the animals induced for EAE with MOG peptide, Ig-MOG reduced the severity of disease while in *trans* treatment with Ig-PLP1 exacerbated the clinical signs of MOG/EAE, where the initial phase of disease was more pronounced and the resolution was delayed. Moreover, prolonged in *trans* treatment with agg Ig-PLP1 did not change the disease exacerbation pattern (Fig. 3) suggesting delayed spreading to PLP1 epitope after completion of the three-injection regimen was not the mechanism responsible for disease exacerbation. In fact, MOG-specific IFN- γ production was evident during exacerbation of disease by agg Ig-PLP1 treatment (Fig. 4). Also, spreading to epitopes other than PLP1 and MOG was excluded because when Ig-PLP1 was accompanied with Ig-MBP3 or Ig-PLP2 exacerbation of EAE persisted (Fig. 6). This was intriguing and led us to suspect involvement of CD8 T cells. Indeed, there are reports in the literature indicating that MOG 35–55 encompasses an epitope recognized by CD8 T cells in the C57BL/6 mouse (18, 19). Because aggregation

of the Igs induces the APCs to produce IL-10, which is a growth factor for CD8 T cells (17), and we found that the anti-MOG response is maintained in agg Ig-PLP1-treated mice compared with untreated, there was a possibility that agg Ig-PLP1 was sustaining activation and expansion of MOG-specific CD8 T cells. However, this postulate proved incorrect as Ag-specific IFN- γ could be inhibited only in the presence of anti-CD4 Ab indicating that CD8 T cells play little or no role in agg Ig-PLP1-mediated exacerbation of MOG-induced EAE (Fig. 5).

In the face of this dilemma, we were left with the possibility that agg Ig-PLP1 may be activating, rather than tolerizing, PLP1-specific T cells, leading to exacerbation of disease. To examine this possibility, agg Ig-PLP1 was combined in a treatment with Ig-PLP-LR, an Ig carrying a PLP1 antagonist (8). The results indicated that agg Ig-PLP-LR reduced the severity of disease during treatment with agg Ig-PLP1 (Fig. 7). Furthermore, when antagonism was conducted before disease induction with MOG peptide, treatment with agg Ig-PLP1 exacerbation of disease was nullified (Fig. 7). This indicates that PLP1-specific T cells are required for exacerbation of disease (Fig. 7). The question then is how these T cells aggravate MOG/EAE. Cytokine screening analysis indicated that in the MOG/EAE mice treated with agg Ig-PLP1 there was production of IL-5 by PLP1-specific T cells that was not observed in untreated or Ig-MOG-treated mice (Fig. 9). Given that IL-5 is a Th2 cytokine usually associated with modulation of autoimmunity, we concluded that it might be indirectly involved in the exacerbation of disease. Knowing that MOG/EAE involves Abs (23, 24), we suspected that IL-5 may be interfering with such Ab responses to aggravate the disease. Surprisingly, however, treatment with agg Ig-PLP1 reduced MOG Ab responses and more specifically the IgG2a and IgG2b isotypes (Fig. 8). Furthermore, neutralization of endogenous IL-5 modulated the disease (Fig. 9) and restored Ab responses with reincrease of IgG2a and IgG2b anti-MOG isotypes to significant serum levels (Fig. 10). Because IL-5-deficient mice manifest signs of EAE similar to wild-type mice (25), it is possible that other cytokine products of Th2 or T regulatory cells contribute to the Ig-PLP1-mediated aggravation of EAE. The fact that disease progression occurs when serum anti-MOG Ab titers diminish and the severity is reduced when the Ab titer increases (by neutralization of IL-5) suggests that the Abs may play a protective role. It has previously been shown that MOG-specific Abs directed against conformational epitopes are pathogenic while those recognizing linear epitopes have no demyelinating effects (26, 27). In this study, because the Abs are induced and detected by the MOG 35–55 peptide, they represent anti-linear epitope Abs that seem to play a protective rather than pathogenic role. Given that MOG is restricted to C57BL/6 haplotype and that this strain is unable to develop pathogenic Abs to conformational MOG epitopes (28), it is likely that the linear epitope-specific Abs contribute protective rather than demyelinating functions. The conclusion that can be drawn from these studies suggests that T cell tolerance in the context of genetic polymorphism could nullify protective humoral responses and aggravate rather than ameliorate autoimmunity.

Overall, Ag-specific therapies are ideally more suitable for treatment of autoimmune disorders than non-Ag-based therapies, presumably because they affect the specific cells responsible for the pathogenesis of the disease. However, complex polymorphisms which could result in unbalanced MHC expression (29) need to be taken into consideration to devise effective Ag-specific therapy against the disease.

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Disclosures

The authors have no financial conflict of interest.

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EXHIBIT 14

Oral Tolerance in Humans

T Cell but Not B Cell Tolerance After Antigen Feeding¹

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The purpose of this study was to investigate whether oral tolerance, defined as Ag-specific immunologic unresponsiveness after Ag feeding, could be induced in humans after prolonged Ag ingestion. Eight adult volunteers ingested a total dose of 0.5 g of keyhole limpet hemocyanin (KLH) followed by subcutaneous immunization with KLH. Eight controls received only the subcutaneous immunization. In the group fed KLH, there was a significant reduction in KLH-specific T cell proliferation ($p = 0.04$) and delayed skin test responses ($p = 0.07$) to KLH. KLH ingestion alone did not induce significant levels of Abs in either serum or secretions. However, after the subsequent subcutaneous immunization, the number of circulating IgG and IgM anti-KLH-producing cells, the titers of serum IgG, IgA, and IgM anti-KLH Abs, and the titers of IgA anti-KLH Abs in saliva and intestinal secretions were significantly greater in the KLH-fed group than in the nonfed group. We conclude that KLH feeding induced systemic T cell tolerance, but B cell priming, at both systemic and mucosal sites. These studies support the concept of using Ag feeding as a treatment for certain immune-mediated diseases. *Journal of Immunology*, 1994, 152: 4663.

The environmental Ags from food and microbial flora are in constant contact with mucosal surfaces and provide a continuous stimulus for the entire immune system. Although a common result of such stimulation is the induction of mucosal and systemic immunity, an alternative outcome is a state of unresponsiveness or tolerance (1, 2). The term oral tolerance refers to a state of systemic unresponsiveness to parenteral immunization that is induced by previous Ag feeding. Oral tolerance of both humoral and cellular immunity has been convincingly demonstrated in rodents fed a wide variety of Ag types (3–8). In several experimental autoimmune diseases, such as experimental allergic encephalomyelitis (9–12), collagen-induced arthritis (13, 14), and experimental autoimmune uveitis (15), autoantigen feeding has blocked

induction of or ameliorated established disease. In some of these models, such Ag feeding has been found to induce CD8⁺ T cells that secrete the cytokine TGF- β upon Ag reexposure in vivo (16, 17). These results have prompted an interest in the feeding of autoantigens as a therapy for human autoimmune diseases (18, 19). However, attempts to induce oral tolerance in some species, such as rabbits, have been unsuccessful (20), and it is unclear whether this approach could be effective in humans, in whom the existence of oral tolerance has not been clearly demonstrated, although suggested (21, 22).

The purpose of this study was to determine whether Ag feeding of humans induces oral tolerance of either the T cell or B cell compartment. We used KLH,⁴ a potent systemic immunogen, because it is a novel Ag to most individuals, and it has been used safely in humans to assess immunocompetence. The strategy was to feed KLH to a group of volunteers; this group and another group not fed KLH were then parenterally immunized, and the ensuing systemic and mucosal immune responses were compared. T cell responses were assessed by a proliferation assay and by skin test reactivity to purified KLH. B cell responses were assessed by ELISA for IgM, IgG, and IgA anti-KLH

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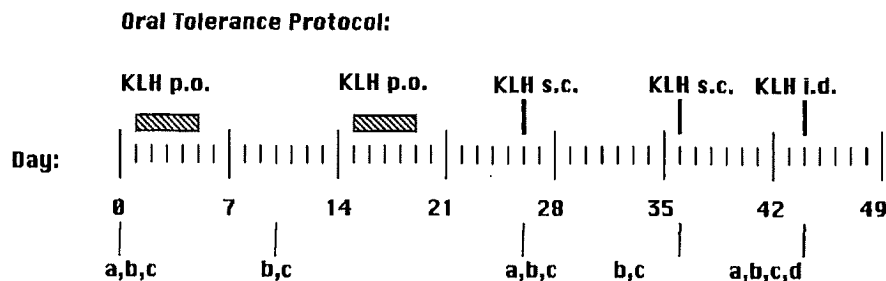
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⁴ Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; ELISPOT, enzyme-linked immunospot; SI, stimulation index; NC, nitrocellulose; SFC, spot-forming cell; EU, endotoxin units.

FIGURE 1. Protocol of feeding, parenteral immunization, and testing used in this project. KLH p.o. = 50 mg of KLH was administered in gelatin capsules per os daily; KLH s.c. = 100 μ g of KLH injected s.c.; KLH i.d. = 10 μ g of KLH injected intradermally as a skin test. Samples for testing were obtained as shown: *a*, peripheral blood cells for ELISPOT and proliferation assays; *b*, serum for ELISA; *c*, intestinal secretions and saliva for ELISA; *d*, delayed skin test responses.



Abs in serum, for IgA anti-KLH Abs in secretions, and by the ELISPOT technique for Ab-producing cells in peripheral blood.

Materials and Methods

Volunteers

A total of 16 healthy volunteers were recruited for the study; 8 of the volunteers (mean age 28 yr, range 23–37; 6 males and 2 females) took part in the study as the experimental group. The other 8 subjects were included in the control group (mean age 27 yr, range 21–36; 7 males and 1 female). The study was approved by the Human Use Committee for the University of Alabama at Birmingham. Informed consent was obtained from each subject before participation.

Experimental design

Fasting volunteers of the experimental group ingested 50 mg of KLH in gelatin capsules on days 1 to 5 and days 15 to 19. They were then immunized s.c. with 100 μ g KLH on day 25 and boosted with the same dose on day 36. The control group underwent the parenteral immunization only (Fig. 1). Samples of blood, saliva, and intestinal secretions were obtained at intervals for assessment of immunity. Blood samples were taken by venipuncture before the start (day 0) and on days 10, 25, 36, and 44 of the study. Secretions were obtained before (day 0) and on days 25 and 44 of the study. An intradermal skin test with KLH (10 μ g) was applied on day 44 and read on days 45 and 46.

Keyhole limpet hemocyanin

KLH as a freeze-dried powder was purchased from Sigma Chemical Co. (St. Louis, MO). For oral use, 50 mg of this preparation was packed into gelatin capsules, which were filled with lactose. For parenteral use, this preparation was dissolved in pyrogen-free saline and passed two times through a polymyxin-agarose column (Boehringer-Mannheim, Mannheim, Germany) at a concentration of 4 mg/ml. This treatment diminished the endotoxin content of the KLH preparation from approximately 1100 EU/ml to below 10 EU/ml as confirmed by Limulus assay (Whittaker, Walkersville, MD). The preparation was filter-sterilized, aliquoted in pyrogen-free saline plus 0.001% merthiolate, and stored at 4°C until use.

Cell isolation and purification

Heparinized blood was diluted 1:2 into Dulbecco's PBS (GIBCO BRL, Grand Island, NY), and the mononuclear cells were isolated by gradient centrifugation over Ficoll-sodium diatrizoate (Organon Teknika, Durham, NC) in 15 ml tubes at 2000 rpm for 20 min, the serum-Ficoll interface was collected and the cells were washed in Dulbecco's PBS followed by RPMI 1640 (GIBCO BRL), supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA), 100 U penicillin/ml and 100 μ g streptomycin/ml. The cells were

counted, adjusted to 5×10^6 /ml, and used directly in the ELISPOT assay. A T cell-enriched fraction was prepared by rosetting with 2-amino-ethylisothionium bromide-treated SRBCs, prepared as described (23) except that the rosetting was allowed to take place overnight on ice. Cells at the plasma-Ficoll interface were removed (E^- cells) and the T cell-enriched fraction (E^+ cells) was prepared by lysis of the SRBCs (24). The E^+ fraction typically contained 60 to 80% of the total number of cells. The E^- cells contained 40 to 50% monocytes as measured by the esterase stain for monocytes/macrophages (25). E^- cells were irradiated at 3000 rad before use as APCs in culture.

T cell proliferation assay

Sterile 96-well microtiter plates (Costar, Cambridge, MA) were used for cell culture. Quadruplicate wells were prepared with adherent APCs by the incubation of 100 μ l of 10^5 E^- cells/ml for 2 h. The wells were washed once with medium and the T cell-enriched (E^+) cells were added at 2×10^6 cells/ml. Replicate wells received KLH (10 μ g/ml), PHA (Sigma Chemical Co., 2 μ g/ml) as a positive control, or medium alone as a negative control. The plates were incubated at 37°C and 5% CO_2 for 2 days for PHA responses and 5 days for KLH-specific responses. The wells were then pulsed with [3H]thymidine (Amersham, Arlington Heights, IL) at 0.5 μ Ci/well for 6 h and harvested on nylon filters; cpm were measured with a liquid scintillation counter. The results were expressed both as a stimulation index (SI), i.e., the ratio of the mean KLH-stimulated cpm divided by the mean unstimulated cpm and as Δ cpm, i.e., the mean KLH-stimulated cpm minus the mean unstimulated cpm.

KLH-stimulated T cell cytokine production

T cells and APCs were cultured with KLH (10 μ g/ml) in wells of 24-well culture plates at 2×10^6 cells/well. After incubation at 37°C and 5% CO_2 for 48 h, the supernatants were harvested and frozen at $-20^\circ C$ until assay. The IFN- γ assay was performed using the WEHI 279 cell line, whose growth is inhibited by IFN- γ (26). TGF- β was measured in a bioassay using the cell line CH-1CAB, whose growth is inhibited by TGF- β (27). A standard curve was constructed for each cytokine and the values for the experimental samples interpolated. In both assays, cell growth was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide method as described (25).

Delayed skin test reactivity to KLH

Endotoxin-free KLH (10 μ g in 100 ml) was injected intradermally at the flexor surface of the forearm using pyrogen-free saline as the control. Induration was measured in mm at 24 h and 48 h by the same observer. A reaction was considered positive if any measurable induration was present.

Enumeration of Ab (spot)-forming cells (SFCs) by ELISPOT

To determine the numbers of IgG, IgA, and IgM anti-KLH-producing cells, the ELISPOT assay was used as described previously (29). Nitrocellulose (NC) 96-well microtiter plates (Millipore Corp., Bedford, MA) were incubated with KLH at a concentration of 20 $\mu\text{g/ml}$ in PBS overnight at room temperature. For the enumeration of isotype-specific Ig-producing cells, the wells were incubated with F(ab')_2 fragments of anti-IgG, anti-IgM, or anti-IgA Abs (Jackson ImmunoResearch Labs, West Grove, PA). Nonspecific protein binding to the NC was blocked by incubation for 2 h with PBS + 10% FCS. The mononuclear cell fraction from the heparinized blood sample was isolated as described above. 100 μl of cell suspension was immediately added in duplicate onto the plate at a range of concentrations from 10^5 to 5×10^6 cells/ml for anti-KLH Ab-producing cells and from 10^4 to 10^6 cells/ml for total Ig-producing cells. After incubation at 37°C for 3 h, the wells were washed three times with PBS and three times with PBS containing 0.05% Tween (Sigma Chemical Co.), and incubated overnight at 4°C with 100 μl of biotin-labeled goat F(ab')_2 anti-IgG, -IgA, and -IgM (Tago, Burlingame, CA) diluted 1:750 in PBS-Tween + 1% FCS. The wells were blotted dry, washed, and developed with Extravidin-alkaline phosphatase (Sigma Chemical Co.) followed by the chromogen substrate. The substrate was prepared by mixing 15 mg 5-bromo-4-chloro-3-indolylphosphate toluidine salt (Bio-Rad Labs, Richmond, CA) and 30 mg p-nitroblue tetrazolium chloride (Bio-Rad Labs), separately dissolved in 1 ml dimethylformamide, and adding the mixture to 100 ml 0.1 M NaHCO_3 + 1 mM MgCl_2 , pH 9.8. The NC plate was blotted dry and the wells were exposed to the chromogen substrate; blue spots appeared, usually within 30 min, where a positive reaction had occurred. When the spots had reached maximal intensity, the plate was rinsed with tap water and allowed to dry. The spots were enumerated under a stereomicroscope at 40-fold magnification. The active synthesis of Ab was confirmed by the incubation of the cells with cycloheximide (25 mg/ml for 2 h), which resulted in 70% to 100% inhibition of SFCs.

Intestinal and salivary secretions

Intestinal secretions and saliva samples were obtained from eight subjects in the fed group and from four subjects in the control group. The intestinal secretion samples were obtained with the use of a polyethylene glycol salt solution Colyte® (Reed & Carnrick, Piscataway, NJ), as described previously (30). Saliva was collected as unstimulated whole saliva by having the subject drool into a centrifuge tube placed in ice. Unstimulated parotid saliva was collected using a Schaefer cup placed over the parotid duct (31). The saliva samples were centrifuged at 10,000 rpm for 3 min in a microfuge to remove debris.

Measurement of Ab by ELISA

Flat bottom polystyrene microtiter plates (Titertek, Flow Labs., McLean, VA) were coated with KLH (10 $\mu\text{g/ml}$ of PBS), blocked with 5% FCS in PBS for 2 h, and washed three times with PBS-Tween. For analysis of serum samples, serum was diluted 1:250 and 1:1000 in PBS-Tween + 1% FCS. For analysis of saliva, the samples were diluted 1:10 and 1:50 in PBS-Tween containing 1% FCS. The plates were incubated overnight at room temperature. For analysis of intestinal secretions, the samples were diluted 1:4 and 1:40 in PBS-Tween + 5% FCS and incubated overnight at 4°C. The washed plates were incubated with biotinylated goat F(ab')_2 anti-IgG (1:8000), anti-IgA (1:1000), or anti-IgM (1:1000) (Tago) for 4 h at 37°C, followed by Extravidin-alkaline phosphatase (1:2000) for 2 h and then developed with para-nitrophenylphosphate substrate (Sigma Chemical Co.). The absorbance was read at 405 nm in a V_{max} kinetic microplate reader (Molecular Devices, Palo Alto, CA). A high titer serum was used as a reference standard and defined to contain 1000 U/ml IgG, 100 U/ml IgA, and 100 U/ml IgM anti-KLH Ab, based on their relative titer in serum. The absorbances of the samples were analyzed and converted into ELISA U/ml by using a computer program based on a four-parameter logistic model. Two control sera were included in each plate; based on the results of these control sera, the intra-assay variation was 3 to 7%, and the interassay variation of anti-KLH Abs was 22% to 26% for IgG, 15% to 26% for IgA, and 14% to 16% for IgM ($n = 26$).

Samples of sera from days 0, 25, and 44 were also tested for Ab to tetanus toxoid and bovine gammaglobulin. Serially diluted sera were tested in Ag-specific ELISA and the Ab concentrations were expressed as ng/ml by referring to calibration curves for each Ig (Ig) isotype, constructed by assaying wells on the same plates coated with anti-Ig isotype Abs and calibrated serum standards.

Total Ig levels in secretions

The amount of IgG, IgA, and IgM in intestinal secretions was assessed by ELISA. Microtiter plates (Titertek, Flow Labs) were coated with affinity-purified goat F(ab')_2 anti-IgG (2.5 $\mu\text{g/ml}$), anti-IgA (5 $\mu\text{g/ml}$), or anti-IgM (2.5 $\mu\text{g/ml}$) in PBS (all from Jackson ImmunoResearch Labs), blocked with 5% FCS in PBS, and washed three times. The samples were added at a dilution of 1:400 and 1:4000 in PBS-Tween + 5% FCS and incubated overnight at 4°C. The reference standards used were purified colostral IgA (2.9 g/l, Ref. 32) and the Monitrol standard (Baxter, McGaw Park, IL) for IgG (9.9 g/l) and for IgM (0.9 g/l). The plates were washed and incubated with biotin-labeled F(ab')_2 anti-IgG, anti-IgA, or anti-IgM (Tago), and developed as above for the Ab determinations.

Statistical analysis

Nonparametric statistical analysis was used. Comparison between groups was performed with the Mann-Whitney U-test for unpaired samples. Comparison within groups was done with the Wilcoxon/Pratt test for comparing two samples and the Friedman test for comparing several samples. The level of significance was chosen as $p < 0.05$.

Results

Induction of T cell tolerance by feeding

T cell proliferation assay. Ag-specific T cell proliferation was assessed before immunization (day 0), after the oral immunization (day 25), and after the parenteral immunization (day 44) (Fig. 2). The SI in both groups was low before the immunization. After the oral immunization, the SI for the KLH-fed group rose moderately but significantly ($p = 0.047$) and at this time point was also higher than the preimmunization value of the control group ($p = 0.007$). However, after the parenteral immunization, the SI in the KLH-fed group was significantly lower than in the nonfed group ($p = 0.04$) (Fig. 2A). Similar results were found when the data are expressed as Δ cpm, with an increase after the feeding in five of eight subjects, followed by a reduction to baseline levels after the parenteral immunization (Fig. 2B). Mean PHA-stimulated T cell proliferation of the KLH-fed group did not change significantly in the KLH-fed vs control groups either after the feeding or after the parenteral immunization, nor in the KLH-fed group before feeding, after feeding, or after parenteral immunization (data not shown).

Delayed skin test response. Intradermal skin testing with KLH was done on day 44 (Table I). At 24 h, only one of eight subjects in the KLH-fed group had a positive reaction, whereas seven of eight in the control group were positive ($p = 0.007$). The same pattern was seen at 48 h, when zero of eight in the fed group were positive vs five of eight in the control group ($p = 0.038$).

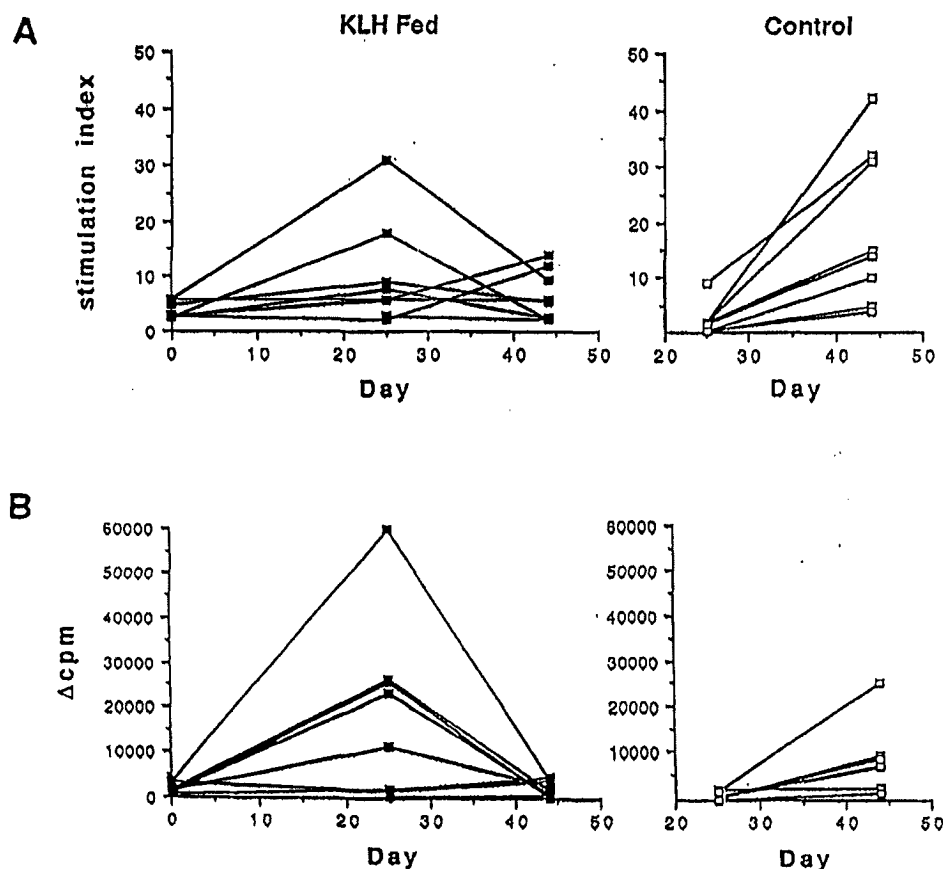


FIGURE 2. KLH-induced proliferation of peripheral blood T cells. The data from each individual shown at day 0 before the study, at day 25 after the oral feeding, and at day 44 after subcutaneous immunization. *A*, stimulation index; *B*, Δ cpm.

Priming of the systemic B cell response by KLH feeding

Anti-KLH SFCs in peripheral blood. Virtually no anti-KLH SFCs were identified in the blood either before or after the KLH feeding. However, IgG, IgA, and IgM anti-KLH SFCs were detected after the parenteral priming and booster doses (Fig. 3). IgG and IgM anti-KLH-secreting cells were significantly greater in number in the KLH-fed group than in the control group only after the parenteral priming on day 36 ($p = 0.028$ and $p = 0.021$, respectively). There were no statistically significant differences on day 44. **Serum anti-KLH Abs.** Feeding with KLH did not produce any detectable serum levels of anti-KLH Abs of any isotype above the background level (Fig. 4). However, KLH feeding did result in the priming of B cells, as demonstrated by a significantly higher serum IgG anti-KLH (Fig. 4A) in the KLH-fed group as compared with controls following the parenteral priming and booster immunization ($p < 0.05$). Both IgA and IgM anti-KLH in the KLH-fed group rose sharply and significantly ($p < 0.01$ for both; Figs. 4, B and C) after the parenteral priming, but were not significantly different from the control group after the parenteral booster.

Serum Abs against a food Ag, bovine gammaglobulin, as well as against tetanus toxoid, were measured in samples obtained at days 0, 25, and 44. No change in mean Ab titer in either the KLH-fed or the control group was found for either of these Ags (data not shown).

Priming of secretory Abs by KLH feeding

Anti-KLH Ab levels in salivary secretions. The IgA anti-KLH levels in both whole saliva (Fig. 5A) and parotid saliva (data not shown) of the KLH-fed group did not increase significantly after the feeding alone, but did increase after the subcutaneous immunization with KLH (day 44) ($p < 0.005$ for whole saliva and $p < 0.015$ for parotid saliva).

Anti-KLH Ab levels in intestinal secretions. No IgG or IgM anti-KLH was detected in the intestinal secretions. IgA anti-KLH levels in intestinal secretions were low, and although they were enhanced after the KLH feeding, this increase was not statistically significant. However, secretory IgA anti-KLH was increased in the KLH-fed group after the parenteral immunization (Fig. 5B) when compared with their own day 0 base line levels ($p = 0.016$).

KLH-stimulated cytokine secretion. On days 0, 25, and 44, T cells were cultured with APC and KLH (10 μ g/ml) for

Table 1. Delayed skin test response to KLH^a

Group	24 h		48 h	
	Mean (range)	Number of positive/total	Mean (range)	Number of positive/total
KLH-fed	1.2 (0–10)	1/8	0 (0–0)	0/8
Controls	11.9 (0–23)	7/8	6.6 (0–20)	5/8
p-value	0.007		0.038	

^a KLH (10 μ g) was injected intracutaneously and the reaction measured as induration (mm) at 24 and 48 h after the injection.

48 h. Supernatants of those cultures were collected and tested for IFN- γ and TGF- β by bioassay. IFN- γ was not detected in any supernatant. TGF- β was detected in a small number of culture supernatants, but there was no relationship to KLH feeding or immunization (data not shown).

Discussion

The present study demonstrates that oral tolerance can be induced in humans; however, with the Ag, dosage, and immunization schedule used, tolerance was limited to the T cell compartment, as demonstrated by a reduction in Ag-specific T cell proliferation and markedly diminished delayed skin test reactivity. Although no anti-KLH-secreting cells or Abs were detected in blood or secretions after the oral feeding, the KLH feeding clearly had an impact on the immune system in that it primed B cells in systemic as well as secretory sites for a greater response upon parenteral immunization. Because the dose, frequency, and type of Ag are known to have a profound influence on the induction of oral tolerance in experimental animals (7, 8), it is possible that varying one or more of these parameters might induce tolerance in B cells in humans as well.

The purpose of this study was to determine whether oral tolerance to protein Ag existed in humans; a detailed examination of the Ag specificity of the tolerance was not planned as a part of the study because it is well established in experimental animals that oral tolerance to proteins is Ag-specific. However, the absence of any effect of KLH feeding on polyclonal T cell proliferation and on serum Ab titers to two common Ags does suggest that oral tolerance is Ag specific in humans as well.

Tolerance of T cells but not B cells has been previously identified after feeding (33, 34) and injecting (35–37) low doses of Ag to animals. A recent study performed in transgenic mice showed that the small amounts of autoantigen released spontaneously in vivo rendered the animals' T cells but not B cells tolerant (38). The B cell system can be tolerized by Ag feeding, but generally requires larger amounts of Ag (4–6). Sensitivity to tolerance induction also varies among T cell subsets. CD4⁺ T cells can be divided into two major subgroups based on their production of cytokines. CD4⁺ Th1 cells produce IL-2 and IFN- γ and mediate delayed hypersensitivity. CD4⁺ Th2 cells produce IL-4, IL-5, IL-6, IL-10 and provide help for B cell

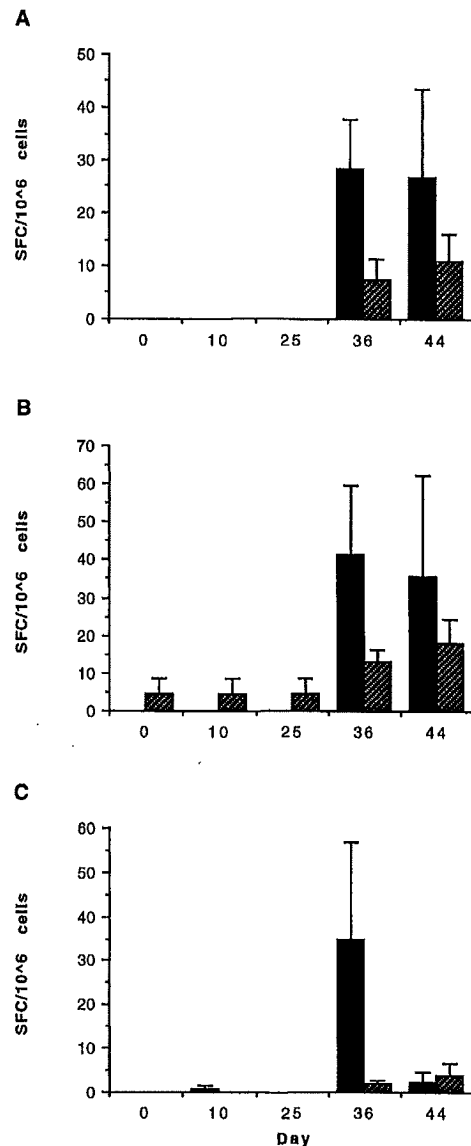


FIGURE 3. Circulating anti-KLH secreting cells (SFC)/10⁶ PBLs of the (A) IgG, (B) IgA, and (C) IgM isotype before and after oral feeding and parenteral immunization with KLH. Solid bars denote the KLH-fed group and lined bars the control group. The data for the control group at days 0, 10, and 25 represent the preimmunization value. Data are shown as geometric means and SE.

Ab responses (39). Parenteral injection of soluble protein Ags into mice tolerized Th1 but not Th2 cells (40); the hyporesponsiveness in Th1 cells was mediated by IL-4 production by Th2 cells (41). There seems to be a gradient of sensitivity to tolerance induction, with Th1 cells > Th2 cells > B cells. Although the existence of the Th1/Th2 subsets is not as well established in humans as compared with mice, the present data are consistent with this paradigm. KLH-specific T cell proliferation and skin test responses are mainly Th1 functions, and these were significantly inhibited by KLH feeding. The B cell priming that

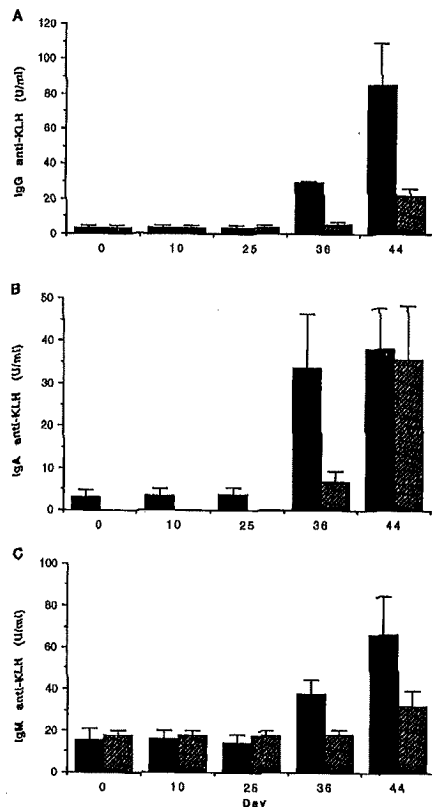


FIGURE 4. Serum Abs to KLH. Anti-KLH of (A) the IgG, (B) the IgA, and (C) the IgM isotype, obtained before the start of the study (day 0), after one period of KLH feeding (day 10), after two periods of KLH feeding (day 25), after parenteral priming (day 36) and after parenteral booster (day 44). The KLH-fed group is denoted by solid bars and the control group by lined bars. For the control group the results presented at days 0, 10, and 25 represent the preimmunization value. Bars represent geometric means and SE.

occurred from the KLH feeding is consistent with a lesser effect of the feeding on Th2 helper cells and B cells. The lack of any Ab production from the feeding alone could result from the dose of Ag being insufficient to trigger Th2 cells or KLH feeding having some inhibitory effect on Th2 cell function.

Tolerance may occur by a number of mechanisms, including clonal deletion, clonal anergy, or suppression. With regard to orally induced tolerance, local and systemic suppressor T cell circuits may be particularly important (5, 9, 42–44). Antigen feeding can generate Ag-specific suppressor T cells in the Peyer's patches of mice, and these T cells later populate systemic lymphoid tissues such as the spleen (42). However, even after suppressor T cells can no longer be identified, the animals remain unresponsive to the fed Ag (43), suggesting the presence of an additional mechanism such as clonal anergy. Ag feeding has been reported to stimulate CD8⁺ T cells that release the immune-inhibitory cytokine TGF- β upon restimulation with

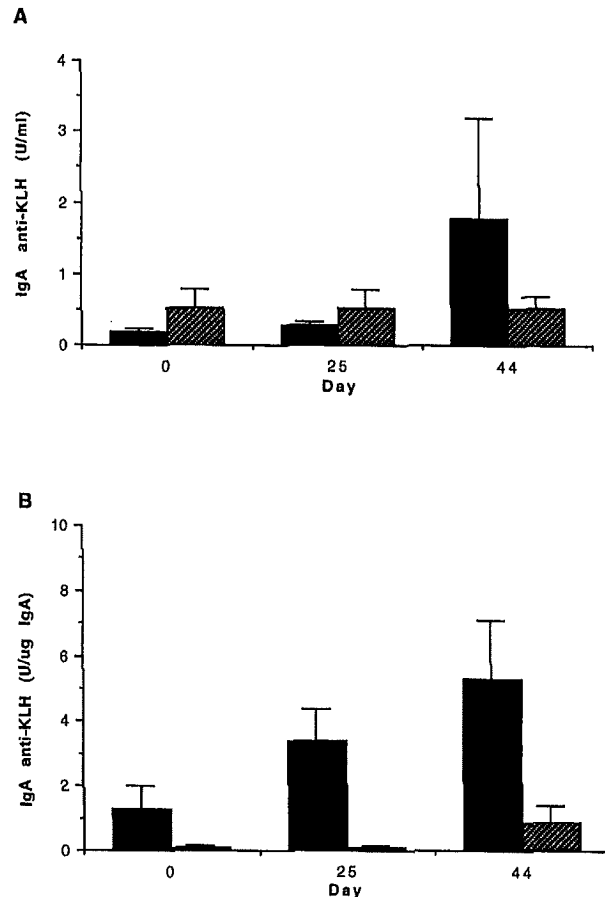


FIGURE 5. IgA anti-KLH Abs in secretions: (A) in whole saliva and (B) in intestinal secretions. Solid bars denote KLH-fed group and lined bars denote control group (four subjects only). For the control group, the results from days 0 and 25 represent the preimmunization value. Data are shown as geometric means and SE.

Ag (16, 17). Accordingly, peripheral blood T cells from KLH-fed and control subjects were restimulated with KLH plus APCs *in vitro*. TGF- β was detected in a small number of samples, but its presence was inconsistent and had no relationship to either the feeding or parenteral immunization with KLH. These data do not exclude a possible role for TGF- β -producing cells, in that the relevant cells may not circulate in sufficient numbers to be detected. Studies detecting them have used lymphoid and other tissues (16, 17), not circulating cells. The role of this or other cytokines, such as IL-4, in oral tolerance remains to be defined.

Although the cellular and molecular mechanisms for the induction of oral tolerance in humans are unknown, oral tolerance may represent an important immunoregulatory process that limits immune response to innocuous food Ags. Certainly, humans ingest food Ags daily in quantities that should result in tolerance, and a small fraction is known to be absorbed into the circulation (45). When

adults with low levels of Ab to BSA were immunized by either ingestion or parenteral injection of BSA, they did not develop an Ab response (21), which may represent a form of oral tolerance to this food Ag. Despite the apparent occurrence of tolerance to food Ags, secretory and serum Abs to them are readily detectable in humans (46–48). The gradient of sensitivity of T cell subsets and B cells discussed above may explain this apparent paradox. Prolonged ingestion of Ag may be sufficient to prime B cells and Th2 cells sufficiently for the production of low levels of Ab.

Despite the large amount of secretory IgA Ab produced daily, it has been difficult to induce secretory IgA responses at mucosal surfaces in any species, including humans, by oral immunization with soluble or nonviable particulate Ags (49). The present results illustrate this, in that even 10 days of Ag feeding did not result in significant Ab production in saliva or intestinal secretions. Thus, these results have implications relative to strategies for oral vaccines. One prediction is that oral-parenteral combinations may be more effective for immunization in humans than the oral route alone. Another possibility is that protein Ags given orally will require mucosal adjuvants to prevent induction of T cell tolerance and to effectively activate both T cells and B cells (49).

These results support the idea that it may be possible to exploit orally induced tolerance in the treatment of human disease such as allergy or autoimmunity. Preexisting IgE responses and delayed hypersensitivity have been successfully down-regulated by Ag feeding in experimental animals, thus providing experimental support to this notion (50, 51). In regard to allergy, hyporesponsiveness to allergens such as tree or grass pollens and leaf extracts has been induced with variable success (52, 53). The induction of T cell tolerance to ingested Ags may be of considerable importance in the amelioration of diseases in which T cells represent the dominant effector mechanism. As mentioned above, oral tolerance to ingested autoantigens has been effectively used in experimental autoimmune diseases (7–13). Whether the feeding of autoantigens to humans can suppress the further progression of autoimmune disease remains to be demonstrated, but clinical studies are under way in multiple sclerosis (54) and rheumatoid arthritis (55). If this can be achieved, the induction of T cell tolerance by Ag feeding may represent a novel form of treatment for autoimmune diseases and hypersensitivity disorders.

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EXHIBIT 15

Oral Tolerance in Humans

Failure to Suppress an Existing Immune Response by Oral Antigen Administration

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ABSTRACT: Orally administered antigens can induce systemic tolerance. In animal models, oral tolerance can both prevent and treat experimental autoimmune diseases. The induction of oral tolerance to human autoantigens has been envisioned as a potential treatment for human autoimmune conditions. The results of previous human studies from our laboratory have provided evidence that oral administration of a model antigen, keyhole limpet hemocyanin (KLH), prior to systemic immunization, can decrease the magnitude of subsequent T cell proliferative and skin test responses to KLH. The present study was designed to test the hypothesis that orally administered KLH could attenuate a preexisting immune response to KLH. Thus, human subjects ($n = 8$) were primed subcutaneously with KLH without adjuvant prior to a 42-day feeding regimen of 100 mg of KLH per day. At the end of the feeding regimen, the subjects were boosted with KLH, again without adjuvant. Eight control subjects were immunized as above with KLH, but fed ovalbumin. The measurement of antigen-driven T cell proliferation, serum and salivary antibody, and dermal delayed hypersensitivity responses to KLH failed to reveal significant differences between subjects fed KLH and those fed ovalbumin. These results indicate that the KLH dose and feeding regimen used in this study failed to attenuate the primary response or to prevent the secondary response to KLH. Therefore, some form of immunomodulation greater than that provided by oral administration of antigen alone is required in humans for suppression of an existing immune response.

KEYWORDS: tolerance; antigen feeding; humans; T cell; antibody

INTRODUCTION

Mucosal tolerance is defined by a marked reduction in systemic immune responses to an antigen previously administered by the mucosal route. This phenomenon

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was demonstrated by Chase in animals fed a contact sensitizing agent and later challenged systemically with the same antigen.¹ Since this pioneering work, many subsequent studies have been performed in animals using a broad spectrum of soluble (e.g., ovalbumin, bovine gammaglobulin, human gammaglobulin) or particulate (e.g., sheep erythrocytes) antigens.² Furthermore, in animal models of autoimmune diseases such as allergic autoimmune encephalomyelitis, collagen type 2-induced arthritis, uveitis, and diabetes mellitus, initial feeding of the autoantigen has prevented disease otherwise induced by systemic immunization with the relevant antigen in adjuvant.² Compared with the large number of experiments performed in animals, basic studies in humans are quite limited. Evidence for the existence of mucosal tolerance in humans was provided in our previous study, in which we administered keyhole limpet hemocyanin (KLH) antigen for 10 days either orally³ or intranasally.⁴ Upon subsequent systemic challenge, cellular immune responses were inhibited, as manifested by decreased T cell proliferation and decreased delayed-type hypersensitivity skin test responses to KLH. In contrast, antibody responses were increased rather than inhibited, with increased levels of KLH-specific antibodies in serum and external secretions, and increased numbers of specific antibody-secreting cells in peripheral blood.

We next examined the immunologic effects of long-term feeding of antigen. For this we used dietary antigen as a surrogate for what might happen if one fed protein antigens long term. To this end, immune responses to the common dietary antigens bovine gammaglobulin (BGG), ovalbumin (OVA), and soybean protein were evaluated in 50 normal human volunteers. Humoral and T cell responses to these antigens were measurable but low, consistent with immune tolerance.⁵ T cell proliferation to dietary antigens was increased significantly by addition of low doses of recombinant human interleukin-2. Peripheral blood mononuclear cells stimulated with BGG or OVA expressed IL-2 receptor α chain, but not IL-2 mRNA, consistent with T cell anergy. In some individuals, T cell proliferation to an unrelated vaccine antigen (tetanus toxoid or purified protein derivative) was suppressed by addition of BGG or OVA, but this inhibition could be reversed with low doses of rIL-2. The conclusion of these studies was that the major mechanism of tolerance to chronic antigen feeding in humans is anergy. We did not find evidence of regulatory T cells or active suppression mediated by inhibitory cytokines such as IL-10 or TGF β .

A number of attempts have been made in humans to use mucosal tolerance in the treatment of human autoimmune disease, with little success to date, as is detailed elsewhere in this volume. Despite this, mucosal tolerance is attractive as a therapeutic modality if it can be translated to humans with autoimmune or chronic inflammatory diseases. However, it remains unknown whether antigen feeding can inhibit a preexisting immune response in humans, which is a requirement for an effective therapy in patients who are already sensitized at the time of diagnosis. It is known in animals that oral tolerance is most effective when the antigen is fed to naive animals prior to systemic immunization. In experimental models of autoimmune disease, antigen feeding in the setting of a preexisting immune response has been much less effective. In the studies reported here, we addressed this question by feeding either KLH or OVA as a control antigen for six weeks to human volunteers who had been primed with a small dose of KLH prior to the antigen feeding.

MATERIALS AND METHODS

Human Subjects and Study Protocol

Sixteen human volunteers (8 males and 8 females), ranging from 22 to 46 years of age, were recruited for this study. The study protocol was approved by the UAB Institutional Review Board, and informed consent form was obtained from all subjects. The volunteers were immunized intramuscularly (i.m.) with 100 µg of endotoxin-free KLH (Pacific Biomarine, Venice, CA) without adjuvant. The antigen-feeding protocol, which consisted of a daily ingestion of 100 mg of either KLH (Calbiochem, La Jolla, CA) or chicken ovalbumin (OVA; Sigma, St. Louis, MO) in gelatin capsules on an empty stomach, commenced on day 7 and ended on day 49. On day 56, all subjects were systemically boosted with 100 µg of endotoxin-free KLH. Delayed-type hypersensitivity (DTH) skin test reactions were measured after intradermal (i.d.) injection of 10 µg of KLH on days 49 and 63. Peripheral blood and saliva samples were collected at the beginning of the study, and on days 7, 28, 49, and 63 thereafter. Peripheral blood was collected by venipuncture with heparin for isolation of cells, or without anticoagulants to obtain serum. Unstimulated parotid saliva was collected with the use of plastic Schaefer cups placed over the opening of the duct of the parotid gland.⁶ After collection, samples were centrifuged and the supernatants aliquoted and frozen at -70°C until assay.

Measurement of Antigen-Specific T Cell Proliferation

T cells were isolated from peripheral blood mononuclear cell fraction (PBMC, obtained by Ficoll-Hypaque gradient centrifugation) by rosetting with 2-aminoethyl-isothiuronium bromide-treated sheep red blood cells.⁷ The nonrosetting cells were irradiated (3000 R) and used as antigen-presenting cells (APC). A mixture of 2×10^5 T cells and 5×10^4 APC suspended in RPMI 1640 supplemented with 5% human AB serum were plated in each well of a 96-well flat bottom plate (Costar, Cambridge, MA). After the addition of 100 µg/mL of antigen (KLH or OVA), the cells were cultured for five days at 37°C, and 5% CO₂. On the fifth day, 0.1 µCi ³H-thymidine was added to each well and the incubation was continued for an additional six hours. Cells were then harvested and T cell proliferation was measured by ³H-thymidine incorporation with a liquid scintillation counter (Beckman, Fullerton, CA).

Measurement of Cytokine Secretion

Isolated T cells were cultured in the presence of irradiated APC at 37°C in a 5% CO₂ for 24 h in complete medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1.5 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin) in the presence or absence of 10 µg/mL KLH or OVA. The supernatants were collected after 24 or 72 h and assayed for levels of IFN-γ, IL-4, IL-10, and TGF-β by ELISA (R&D Systems, Minneapolis, MN).

Measurement of Skin Delayed-Type Hypersensitivity Responses

The diameter of induration and erythema was measured 24 h after the volunteers were challenged intradermally with 10 µg of KLH on days 7, 49, and 63.

Measurement of B Cell Responses by ELISPOT Assay

The number of KLH- and OVA-specific as well as total antibody-secreting cells (ASC) was determined by enzyme-linked immunospot assay (ELISPOT), as described by Czerkinsky⁸ and previously used by our group for a similar study.³ Because of the high degree of variability among the volunteers in the number of ASC per 10⁶ PBMC, we expressed the KLH- or OVA-specific ASC as percentage of total isotype.

Measurement of Antibody Responses in Serum and Secretions

Antibodies were measured by ELISA as previously described.⁵ Quantitation of specific and total levels of antibodies was accomplished by ELISA. Ninety-six-well polystyrene plates (Nalge Nunc International, Roskilde, Denmark) were coated with the same concentrations of antigens or antibodies as those used for ELISPOT assay. Serial dilutions of samples or standards (100 μ L/well) were added to plates blocked with 5% heat-inactivated FCS in PBS-Tween 20 and then incubated overnight at 4°C. The standards consisted of a pool of human sera calibrated against WHO standards (Moni-Trol, Dade International, Miami, FL) and, for saliva, of purified colostrum S-IgA prepared in our laboratory. The same biotin-labeled reagents as those used for the ELISPOT assay were added to the plates and incubated for three hours at 37°C. After another hour, incubation with ExtrAvidin peroxidase-conjugate (Sigma), the peroxidase substrate, *O*-phenylenediamine-H₂O₂ (Sigma), was added, and the color reaction was stopped with 1 M sulfuric acid. The absorbance was measured in an EL312 Bio-Kinetics microplate reader (Bio-Tek Instruments Inc., Winooski, VT) at 490 nm. The results were calculated by interpolation of optical densities on calibration curves constructed using the Delta Soft 3 computer program. Antigen-specific and total immunoglobulin levels were calculated by interpolating the optical densities on calibration curves, as previously described⁹ using the DeltaSoft 3 program (BioMettalics, Inc., Princeton, NJ).

Statistical Analysis

Data are expressed using descriptive statistics such as mean and standard deviation. A nonparametric two-tailed test (Wilcoxon or Mann-Whitney) was employed to determine the significance between the two groups, and a paired *t* test was used to test the significance in the same group at different times.

RESULTS

Antigen-Specific T Cell Proliferation

The effect of prolonged KLH or OVA ingestion on the KLH-specific T cell responses of the intramuscularly KLH-primed individuals was tested by an *in vitro* proliferation assay. T cell proliferation of KLH-fed volunteers increased during the feeding and particularly 1 week after the systemic boost at day 56. Importantly, no significant differences were detected between the group of volunteers fed KLH or

those fed OVA (FIG. 1). The OVA-specific T cell proliferation was modest, and even in the OVA-fed group, there was very little increase over the baseline values.

DTH Responses

Skin testing was used to determine whether the DTH mediated by KLH-sensitized CD4 T lymphocytes could be abolished or diminished by KLH feeding. As shown in TABLE 1, the prolonged ingestion of large amounts of KLH by KLH-primed

TABLE 1. Skin test responses to KLH

Volunteers Ingesting:	Day 8 ^a	Day 50 ^a	Day 64 ^a
KLH	2.0 (0-5)	10 (0-40)	17 (0-60)
OVA	1.3 (0-3)	15 (10-30)	14.5 (1-30)

^aNone of the differences were statistically significant. Data represent mean and range (in parentheses) of induration diameter expressed in millimeters.

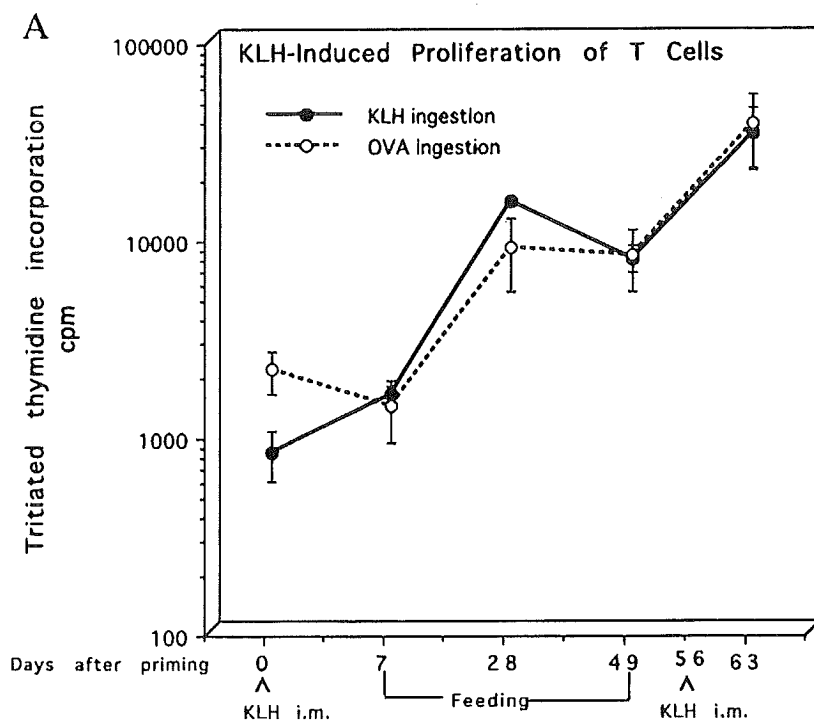
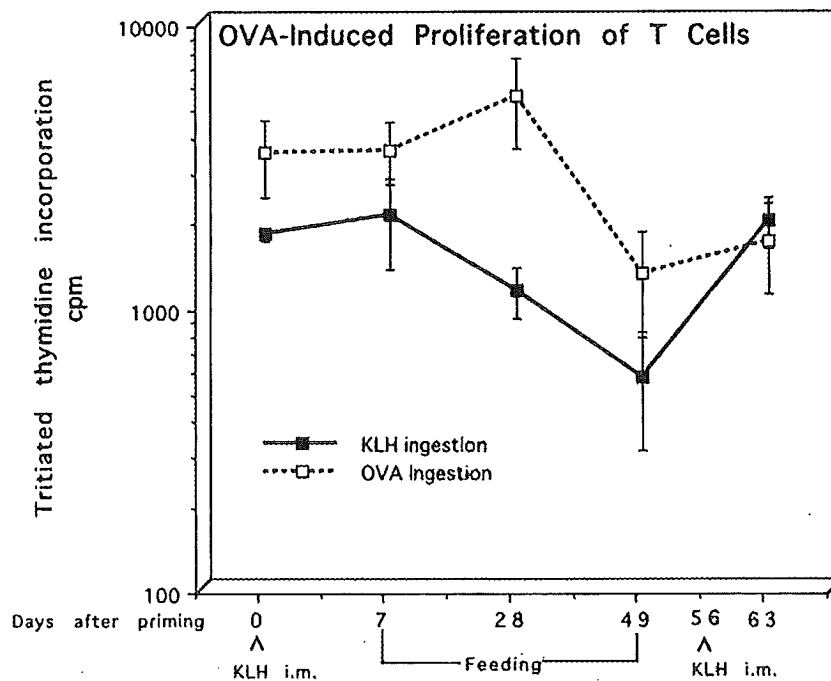


FIGURE 1. Antigen-induced proliferation of T cells isolated from peripheral blood of volunteers before and after systemic priming with KLH, after ingestion of either KLH ($n = 8$ individuals) or OVA ($n = 8$ subjects), and following intramuscular challenge with KLH. Proliferative responses to KLH (A) and OVA (B) were measured by ^3H -thymidine incorporation and expressed as mean Δ cpm (stimulated-unstimulated) (\pm SD).

B

FIGURE 1 — *continued*.

individuals did not affect their DTH reactions. These data complement the antigen-stimulated T cell proliferation data, and demonstrate that oral tolerance, manifested as reduced T cell responsiveness, was not induced on the background of a preexisting systemic response.

Secretion of Cytokines by T Cells after in Vitro Stimulation with Antigen

Cells isolated from the peripheral blood of volunteers after the systemic challenge were cultured with KLH, OVA, or the mitogen ConA (as a control), and cytokines (see Methods) secreted in culture supernatants were measured. An increase in secreted IL-2 was detected after KLH stimulation of cells; however, the difference between the KLH- and OVA-fed volunteers was not statistically significant. Likewise, IFN- γ , TGF- β , and IL-4 secretion were not significantly different between the two groups. A small increase in secreted IL-10 was measured in KLH-stimulated cells isolated from KLH-fed volunteers, but it was not statistically significant (data not shown).

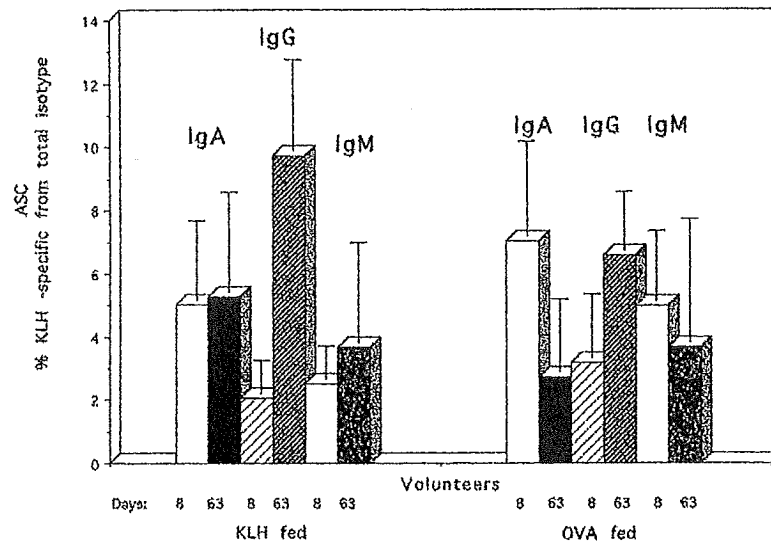


FIGURE 2. ELISPOT assay of IgA, IgG, or IgM isotypes from KLH-specific antibody-secreting cells (ASC) in peripheral blood of volunteers fed KLH or OVA, from samples taken one week after each intramuscular injection with KLH. The bars represent mean percentage of total IgA, IgG, or IgM ASC secreting anti-KLH antibodies (\pm SD).

Peripheral Blood ASC to KLH or OVA

The percentages of cells secreting KLH- or OVA-specific antibodies as a percentage of the total IgA, IgG, or IgM isotypes are presented in FIGURE 2. One week following the priming injection with KLH of the 16 volunteers, 1% or more of total isotype of ASC was KLH specific: IgA in 12 volunteers (more than 5% in 7 volunteers), IgM in 14 (more than 5% in 6), and IgG in 9. The response against OVA was low, not exceeding 0.14% of total IgA-, 0.23% of the IgG-, and 1.49% of IgM-secreting cells. The OVA feeding did not increase the number of peripheral blood lymphoblasts secreting anti-OVA antibodies, and the OVA-specific IgM-secreting cells were not detectable. One week after the booster challenge with KLH of volunteers fed with either KLH or OVA (day 63), the proportion of cells secreting KLH-specific antibodies was not statistically significantly different between the two groups, nor was the difference significant between the percentages measured after the booster compared with after the primary immunization (FIG. 2). After the systemic challenge, the percentage of cells secreting IgA antibodies against KLH was decreased in OVA-fed volunteers, consistent with a stimulatory effect on B cell responses, although this difference did not reach statistical significance.

Levels of KLH-Specific Antibodies in Sera and in Saliva

The measurement of antigen-specific and of total immunoglobulin isotypes indicated that systemic priming with KLH resulted in the induction of antigen-specific

A

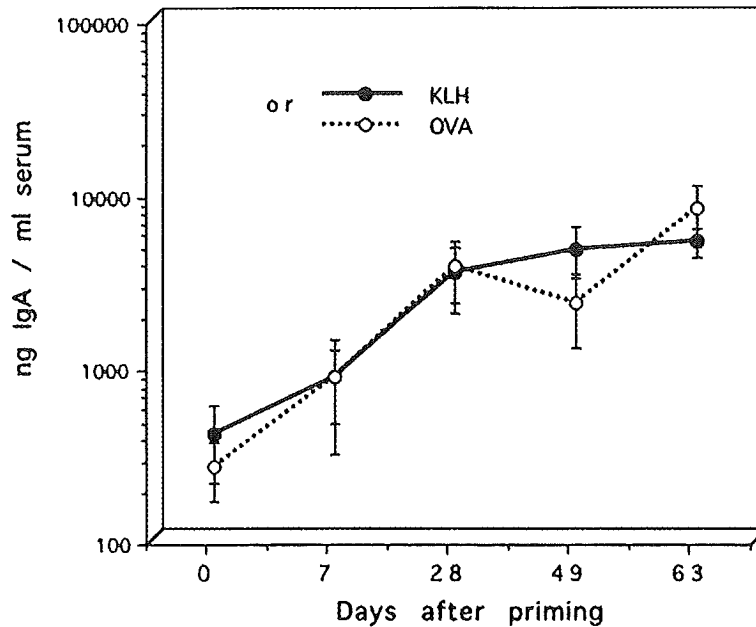
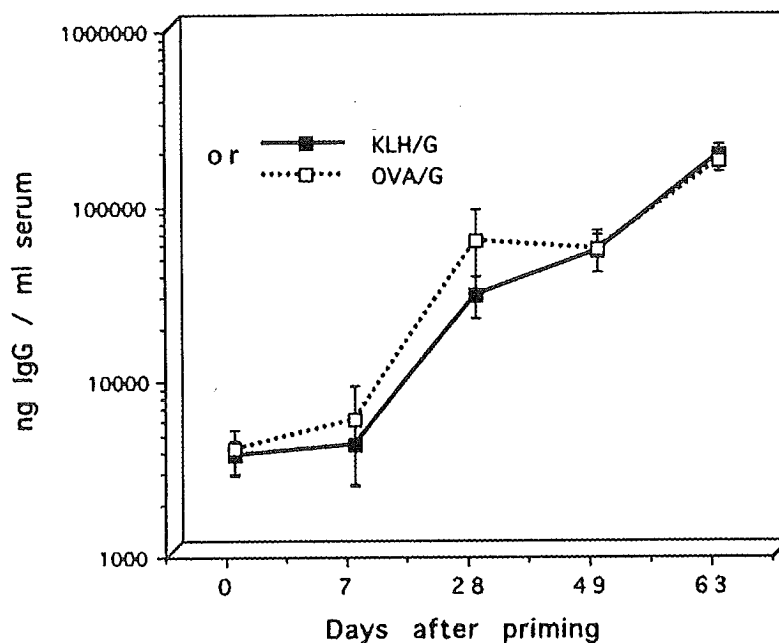


FIGURE 3. KLH-specific IgA (A) and IgG (B) antibodies in sera collected from volunteers enrolled into this study. At each time point, the values represent the mean (\pm SD) of the ELISA data generated from the eight volunteers in the experimental (fed KLH) or control (fed OVA) groups.

antibodies in sera of all 16 participants in this study. After feeding, KLH-specific IgA increased in sera of three out of the eight volunteers fed KLH, and decreased in the other five, as well as in all volunteers who ingested OVA capsules. The subcutaneous challenge with KLH increased the anti-KLH IgA titers in all individuals who ingested OVA and in four of the KLH-fed volunteers (FIG. 3a). IgG anti-KLH increased in all 16 volunteers (FIG. 3b). Little or no increase in IgM titers was observed in either KLH- or OVA-fed groups (data not shown). None of the changes were statistically significant. Neither feeding nor systemic injection with KLH induced statistically significant changes in salivary KLH-specific IgA.

B

FIGURE 3 — *continued.*

DISCUSSION

These results demonstrate that extended oral administration of KLH to individuals with preexisting immunity to it failed to induce a state of tolerance, as manifested by diminished T cell proliferative responses or delayed hypersensitivity skin test responses. Both of the latter have previously been demonstrated in naive human volunteers fed KLH at similar doses. These results confirm and extend studies previously performed in animals and in humans. Chase,¹ using oral feeding, was unable to suppress cellular responses in animals previously sensitized to the antigen that was subsequently fed. Czerkinsky *et al.*¹⁰ were unable to improve the clinical status or to arrest the development of collagen type 2 arthritis or autoimmune allergic encephalomyelitis by mucosal administration, either oral or nasal, to animals sensitized systemically two weeks before the mucosal antigen exposure. In humans, oral administration of extracts of poison ivy or poison oak failed to suppress cutaneous

delayed-type hypersensitivity reactions in previously sensitized individuals and, for lack of efficacy, were abandoned.^{11,12} As mentioned above, antigen feeding in experimental models of autoimmune disease is much more effective when antigen is given prior to systemic immunization rather than after. These results are consistent with the negative results of the multiple clinical trials, reported elsewhere in this volume, in which autoantigen feeding of patients with various diseases has failed to provide any clinical efficacy. These present data plus those of the negative clinical trials indicate that simple antigen feeding by itself is unlikely to be an effective therapeutic modality in patients.

From experiments in animals it is clear that oral tolerance is a complex phenomenon and that multiple factors influence its development, including the dose of antigen, frequency of feeding, the route of administration, the immunogenicity of the antigen, the age of the animals, and the genetic background of the individual.² These multiple variables make comparison of results difficult, even in rodents. There appears to be an early and a late phase of oral tolerance, and each probably has different mechanisms. Clearly, oral tolerance is not one entity, and multiple mechanisms exist, including deletion, anergy, and the induction of regulatory cells, all of which are probably operating simultaneously to varying degrees.

In contrast to the rich data in experimental animals, particularly in mice, there is a dearth of basic immunology studies on oral tolerance in humans. Most experiments performed in mice are carried out in inbred strains that are genetically uniform. These mice represent, in essence, monoclonal reagents, and results in a given strain would be equivalent to the reactivity that would be expected from a single person. Humans have very diverse responses to immunization and antigen feeding,⁵ and overall their oral tolerance response appears to be less than that seen in inbred strains of mice. That may be more a reflection of the genetic diversity of humans relative to the inbred strains than anything else. It is clear that oral tolerance is an active form of immune response and not simply the absence of a response. Thus, the effective use of oral tolerance to treat human disease will require adjuvants and delivery systems, just as is required for oral immunization. Adjuvants and delivery systems that enhance oral tolerance have already been described in experimental systems.^{10,13,14}

If mucosal tolerance as a therapeutic modality is to advance, where does it go from here? Clearly, the development of adjuvants and delivery systems to enhance tolerance are needed and should be a high priority. In humans, the induction of anergy to a single antigen is unlikely to be beneficial, and thus studies should target the induction of regulatory cells that are able to mediate bystander suppression. The latter has been demonstrated clearly in multiple experimental models,¹⁵⁻¹⁹ although not yet demonstrated in humans. Systems are available to test for bystander suppression in humans⁵ and could be applied after antigen feeding. Clearly, more studies on the mechanisms involved in humans, and, for that matter, in mice, are needed, particularly studies dissecting early versus late events. Dose is a critical variable in rodent models but has been chosen fairly arbitrarily in human trials. Dose-ranging studies on volunteers or individuals with a given disease are crucial and should be done prior to large clinical trials or antigen feeding. The notion of exploiting mucosal tolerance as a therapeutic approach to human disease remains attractive, and the initial trials attempting simple antigen feeding were worthwhile. However, the negative results are clearly telling us that the situation in humans with various diseases and preexisting immunity is quite complex, and new approaches are needed.

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EXHIBIT 16

INDUCTION OF IMMUNE TOLERANCE TO HUMAN TYPE I COLLAGEN IN PATIENTS WITH SYSTEMIC SCLEROSIS BY ORAL ADMINISTRATION OF BOVINE TYPE I COLLAGEN

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Objective. To determine whether oral tolerance to type I collagen (CI) could be induced in patients with systemic sclerosis (SSc).

Methods. Twenty adult patients with limited or diffuse SSc were enrolled in a study to receive 0.1 mg of solubilized native bovine CI daily for 1 month, followed by 0.5 mg daily for 11 months. Peripheral blood mononuclear cells (PBMC) were obtained from the patients and cultured with human $\alpha 1(I)$ and $\alpha 2(I)$ chains, before and after CI treatment. Culture supernatants were analyzed for levels of interferon- γ (IFN γ) and interleukin-10 (IL-10). Sera obtained before and after treatment were analyzed for levels of soluble IL-2 receptor (sIL-2R). Although this study was not intended to assess the clinical efficacy of oral CI administration in SSc, selected measures of disease severity and organ involvement were evaluated.

Results. Oral administration of CI to SSc patients induced significant reductions in levels of IFN γ and IL-10 in $\alpha 1(I)$ - and $\alpha 2(I)$ -stimulated PBMC culture supernatants, indicating that T cell immunity to CI was decreased by this treatment. Serum levels of sIL-2R also decreased significantly after oral CI treatment, suggesting a reduction in T cell activation. Significant improve-

ments occurred in the modified Rodnan skin thickness score and the modified Health Assessment Questionnaire after 12 months of oral CI in this open trial. The lung carbon monoxide diffusing capacity improved statistically and showed a trend toward clinically significant improvement.

Conclusion. Oral administration of bovine CI to patients with diffuse or limited SSc induces a reduction in T cell reactivity to human CI, appears to be well tolerated, and does not worsen the disease. Further evaluation of oral tolerance to CI in patients with SSc is justified to determine whether it has therapeutic efficacy.

Type I collagen (CI) is the most abundant of all collagens in humans (1). It is present in blood vessels, skin, lungs, heart, kidneys, and intestines, all of which are affected in systemic sclerosis (SSc) (1). CI is a heterotrimer molecule composed of two identical $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain (1). Each α chain contains 1,014 amino acid residues (1). Human and bovine CI have ~92% homology at the amino acid level (2,3). Evidence for cellular immunity to CI in SSc patients was first demonstrated by our group in 1976 (4) and has been confirmed by other investigators (5,6). We found that peripheral blood mononuclear cells (PBMC) from 92% of SSc patients produce chemotactic cytokines when cultured with CI, whereas only 8% of PBMC from healthy subjects do so (4). Hawrylko et al (5) also showed that peripheral blood CD4⁺ T cells from patients with SSc produce interleukin-2 (IL-2) in a dose-dependent manner in response to stimulation with human CI, while those from healthy subjects do not.

A major portion (approximately one-third) of the body's immune cells reside in the gut-associated lymphoid tissue (GALT) (7). The GALT is particularly effective in mounting a tolerogenic response to ingested soluble proteins (7,8). This process, called oral toler-

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ance, has been repeatedly demonstrated in laboratory animals. For example, when mouse strains susceptible to experimental allergic encephalomyelitis (EAE) after systemic immunization with myelin basic protein (MBP) are fed MBP prior to immunization, they develop less EAE or no EAE compared with placebo-fed MBP-immunized controls (9).

The mechanisms that mediate oral tolerance include active cellular suppression (regulatory T cells), clonal anergy, and clonal deletion (10–12). The particular dose of antigen and the frequency of feeding determine which mechanism(s) predominates (10,12). Multiple oral feedings of low-dose soluble antigen favor development of regulatory CD4⁺ T cells that secrete Th2 cytokines, such as IL-4 and IL-10, and transforming growth factor β 1 (TGF β 1)-secreting T cells (Th3 cells) (10,12). These regulatory T cells migrate to peripheral sites throughout the body, and when they encounter the antigen to which they are tolerized, they collectively secrete IL-4, IL-10, and TGF β 1, which can down-regulate Th1 CD4 cells reacting to a variety of antigens, a process called “bystander suppression” (10,13,14).

Since many of the antigens that are involved in human autoimmune diseases are unknown, it is theoretically possible that, by feeding low doses of antigen from the organs or tissues that are the target of autoimmune attack, T cell responses to other autoantigens perpetuating the disease can be down-regulated. CI qualifies as a candidate oral tolerance antigen in SSc, in that it is present in all of the target organs. Since most SSc patients exhibit sensitization to CI (1,4–6), as manifested by cytokine production by PBMC during culture with CI or constituent α 1 and α 2 chains, successful tolerization to CI after it has been orally administered to SSc patients can be assessed by determining whether there are decreases in cytokine production by PBMC cultured with CI α chains. The present phase I study was undertaken to determine whether daily administration of oral bovine CI to patients with SSc would result in down-regulation of the immune response to human CI.

PATIENTS AND METHODS

Patient recruitment and characteristics. This study was approved by the Institutional Review Board at The University of Tennessee Health Science Center. Patients were recruited from University of Tennessee and community rheumatology practices in Memphis. Inclusion criteria were as follows: age \geq 18 years; diagnosis of limited or diffuse SSc by the criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) (15); and patient's PBMC demonstrated reactivity to bovine CI, as

defined by the production of IL-10 (\geq 2 times baseline production) when cultured with native bovine CI. Patients taking D-penicillamine, captopril, or calcium channel blockers were required to be receiving stable doses of these agents for at least 3 months prior to enrollment. The maximum allowable dosage for D-penicillamine was 750 mg/day. Patients taking corticosteroids were required to be receiving a stable dose for at least 1 month prior to enrollment; the maximum allowable dosage was 10 mg/day of prednisone equivalent.

Patients were excluded from the study for the following reasons: inability to render an informed consent in accordance with institutional guidelines; receiving another investigational drug (excluding D-penicillamine) within 90 days of study initiation; a concurrent serious medical condition that, in the opinion of the investigators, made the patient inappropriate for the study; an SSc-like illness associated with environmental, ingested, or injected agents, such as L-tryptophan, tainted rapeseed oil, vinyl chloride, or bleomycin; morphea, linear scleroderma, or eosinophilic fasciitis; a positive pregnancy test; use in the previous 3 months of cyclophosphamide, cyclosporin A, methotrexate, or azathioprine; allergy to beef; or malabsorption syndrome.

Design and duration of the study. The study was an open-label trial to determine whether oral CI treatment would down-regulate PBMC cytokine production when cultured with α 1(I) and α 2(I). Patients received 0.1 mg/day of solubilized bovine CI for 1 month, followed by 0.5 mg/day for 11 months. Collagen was solubilized in 0.1M acetic acid and aliquoted into individual-dose vials. Patients kept the vials refrigerated. Each morning, the patient added 1 vial of the CI preparation to 4–6 ounces of cold orange juice and drank it just before eating breakfast. Patient compliance was monitored by counting the numbers of empty and full vials returned at each visit.

Concomitant medication. Patients were not allowed to increase dosages of D-penicillamine, captopril, calcium channel blockers, or corticosteroids during the study. Patients were dropped from the study if increases in any of these medications were deemed medically necessary by their primary physicians.

Clinical measurements. Significant clinical responses were not expected due to the small study size and the variability in disease classification, manifestations, and duration. However, the following measures of disease severity and organ involvement were evaluated: modified Rodnan skin thickness scores (MRSS) (16) at 0, 1, 2, 3, 6, 9, and 12 months; pulmonary function tests (PFTs; spirometry) with measurement of the diffusing capacity for carbon monoxide (DLCO; performed by the same personnel using the same equipment) at 0, 3, 6, 9, and 12 months; serum creatinine levels at 0, 1, 3, 6, 9, and 12 months; and the modified Health Assessment Questionnaire (M-HAQ) (17,18) at 0, 3, 6, 9 and 12 months.

Microculture of SSc PBMC with α 1(I) and α 2(I). Briefly, before and after 3, 6, and 12 months of oral bovine CI treatment, PBMC were isolated by Ficoll-Hypaque density-gradient centrifugation and set up in culture in 48-well tissue culture plates (2×10^6 cells in 0.5 ml of RPMI 1640 containing 100 units/ml of penicillin, 100 μ g/ml of streptomycin, 2 mM L-glutamine, 20 mM HEPES, and 5% fetal calf serum). Cultures were set up with 50 μ g/ml each of purified bovine α 1(I) and α 2(I) chains, and phytohemagglutinin (PHA; 10 μ g/ml) and phosphate buffered saline (PBS) as controls in duplicate wells. After 5 days of culture, supernatants from duplicate

wells were pooled, harvested by centrifugation, and frozen at -70°C until assayed for cytokine levels (within 30 days).

Measurement of cytokines in serum and PBMC supernatants. After screening for several cytokines in supernatants from SSc PBMC cultured with $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$, we found that most SSc patients produced increased amounts of interferon- γ (IFN γ) and IL-10 protein, as measured by enzyme-linked immunosorbent assay (ELISA). These cytokines were subsequently measured in all culture supernatants.

IFN γ and IL-10 levels were measured by commercial ELISA (R&D Systems, Minneapolis, MN) in supernatants harvested from microcultures of SSc PBMC stimulated by PHA, $\alpha 1(\text{I})$, and $\alpha 2(\text{I})$, and PBMC plus PBS as a control for background cytokine production. A positive response to $\alpha 1(\text{I})$ or $\alpha 2(\text{I})$ was arbitrarily defined as IL-10 or IFN γ levels in $\alpha 1(\text{I})$ - or $\alpha 2(\text{I})$ -stimulated PBMC culture supernatants that were ≥ 2 times the respective cytokine level in the PBMC plus PBS control supernatant. Soluble IL-2 receptor (sIL-2R) levels were measured by ELISA (R&D Systems) in sera obtained before and after 12 months of oral CI treatment. All samples were tested in duplicate.

Measurement of T cell subsets by flow cytometry. Isolated PBMC obtained from samples taken at 0 and 6 months of oral CI treatment were reacted with a panel of monoclonal antibodies that recognize T cell-specific markers: CD4+, CD8+, CD3+DR+, CD4+CD45+RA+, CD4+CD45+RO+, CD8+CD45+RA+, CD8+CD45+RO+, and CD4+CD26+ and analyzed by fluorescence-activated cell sorter at the University of Tennessee Molecular Resource Center.

Preparation and handling of bovine CI. Bovine CI was prepared as previously described (19). Bovine fetuses from pregnant cows were obtained from a local slaughterhouse within 1 hour of death. The skins of 4 fetal calves were removed and maintained at 4°C throughout the preparation. The tissue was sliced into strips and processed through a household meat grinder, then homogenized in a Waring blender with ice chips. The homogenate was centrifuged (10,000g) for 30 minutes and reextracted twice with 1M NaCl (pH 7.6, with 0.05M Tris HCl) and twice with 0.1M acetic acid to remove some type III soluble collagen and much of the noncollagenous components. The final pellet (~ 500 gm) was suspended in 16 liters of 0.1M acetic acid, and the pH was adjusted to 2.8 with formic acid.

Type I collagen was solubilized by overnight (16 hours) digestion with 20 gm of pepsin ($3\times$ crystallized; Sigma, St. Louis, MO) at 4°C . The digest was centrifuged (10,000g for 30 minutes), and the insoluble pellet was discarded. Type I collagen in the supernatant was precipitated by addition of 5M NaCl solution to a final concentration of 0.8M. This was centrifuged as before, and the pellet was redissolved in 0.1M acetic acid. The pH was adjusted to 7.4 with 0.05M Tris and 10M NaOH to inactivate pepsin. Solid NaCl was added to a concentration of 1M, and the solution was centrifuged. The supernatant was collected, and the NaCl content was increased to 1.7M with 5M NaCl. This was centrifuged to remove contaminating CIII. The 1.7M NaCl supernatant was further adjusted to 2.5M NaCl, which precipitated the CI.

The CI pellet was collected by centrifugation and redissolved in 0.5M NaCl, 0.05M Tris, diluted to 0.2M NaCl with water, and 50 gm of DE-52 was added to create a slurry.

This was stirred overnight and centrifuged to remove any DEAE that had bound any remaining pepsin and contaminating glycosaminoglycans. The supernatant was dialyzed against 0.02M NaH_2PO_4 to precipitate CI. The pellet was redissolved in 0.01M acetic acid, dialyzed exhaustively against the same, and stored at -80°C until used.

The homogeneity of the CI was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which showed an $\alpha 1(\text{I})/\alpha 2(\text{I})$ ratio of 2:1 with no contaminating type V or type III collagen. The $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$ collagen chains were separated by carboxymethyl cellulose chromatography, and constituent α chains were digested with cyanogen bromide (19).

Frozen CI stock containers were allowed to thaw over 2–3 days at 4°C prior to dispensing into vials. Thawed collagen was centrifuged at 4°C at 12,000g to remove particulates. The collagen was diluted to a concentration of 50 $\mu\text{g}/\text{ml}$ or 250 $\mu\text{g}/\text{ml}$ with cold (4°C) 0.1M acetic acid. The diluted CI was filtered at 4°C through a glass fiber Acrodisc (Gelman Sciences, Ann Arbor, MI) and then a 0.45 μ filter (Nalgene filter; Nalge, Rochester, NY) and aliquoted (2 ml) into sterile 2-ml screw-top polypropylene vials (Nalgene vials; Nalge). Vials were placed in plastic bags (35 vials/bag) and stored frozen at -20°C until given to the patients.

Statistical analysis. Cytokines produced by PBMC in response to culture with bovine CI, serum levels of sIL-2R, results of PFTs, and clinical variables were analyzed by Student's paired *t*-test to determine whether significant changes occurred after 3, 6, 9, or 12 months of oral CI treatment, compared with pretreatment values. Correlations of the M-HAQ or the MRSS versus IL-10 or IFN γ were analyzed by Spearman's correlation test.

RESULTS

Patient characteristics at study entry. Twenty-five patients with SSc were screened. Twenty-four exhibited production of IL-10 or IFN γ that was ≥ 2 -fold higher than the levels in parallel cultures of the patients' PBMC plus PBS but without $\alpha 1(\text{I})$ or $\alpha 2(\text{I})$. Four patients had complications of SSc or other medical diseases that disqualified them from the study. One patient was enrolled but withdrew from the study very early. The characteristics of the remaining 19 patients are shown in Table 1.

The patients were predominantly female, and the majority had late, diffuse disease. All patients satisfied the ACR preliminary criteria for the classification of SSc. Thirteen were white and 6 were African American. Only 3 patients had a disease duration of < 2 years. Only 3 patients were currently taking D-penicillamine. The 5 patients taking nonsteroidal antiinflammatory drugs (NSAIDs) discontinued these during the last 6 months of CI treatment. Three patients took 5 or 10 mg/day of prednisone throughout the study period.

Side effects, withdrawals, and compliance. Seventeen patients were treated for 12 months. Two patients dropped out because of difficulty with transportation: one

Table 1. Characteristics of the patients taking oral type I collagen for 1–12 months*

Sex	
Female	15
Male	4
Race	
White	13
African American	6
SSc type	
Diffuse SSc	14
Limited SSc	5
Age, mean \pm SD years	50.7 \pm 2.6
Disease duration	
Mean \pm SD years	9.1 \pm 2.0
<2 years' duration	3
Medication use	
Penicillamine	3
NSAIDs	5
Prednisone	3

* Except as noted otherwise, values are the number of patients. SSc = systemic sclerosis; NSAIDs = nonsteroidal antiinflammatory drugs.

very early (<1 month), the other after 6 months. One patient developed a foot drop of uncertain etiology and was removed from the study after 6 months of therapy. No other possible side effects were noted. There was 100% compliance by each patient until the time each dropped out of the study or the study was completed.

Induction of T cell tolerance to CI by administration of oral CI. The daily administration of bovine CI for 12 months was accompanied by significant reductions in IFN γ production by PBMC cultured with purified α 1(I) and α 2(I) chains of human CI as measured after 6 and 12 months of treatment (Figure 1A). IFN γ is a Th1 cytokine, and its reduced production by α 1(I)- and α 2(I)-stimulated PBMC suggests that oral tolerance to CI was effected. Quite surprisingly, IL-10 levels in the same PBMC culture supernatants were also significantly reduced after 3, 6, and 12 months of oral CI treatment (Figure 1B). The production of IFN γ and IL-10 by PBMC stimulated with PHA was not statistically different before or at 3, 6, or 12 months after oral CI treatment (results not shown).

After 12 months of oral CI treatment, there was a significant reduction (as determined by Student's paired *t*-test) in the serum levels of sIL-2R (Figure 2).

T cell subsets measured by the following markers did not change after treatment with oral CI treatment: CD8+, CD4+, CD3+DR+, CD4+CD45+RA+, CD4+CD45+RO+, CD4+CD26+, CD8+CD45+RA+, and CD8+CD45+RO+ (results not shown).

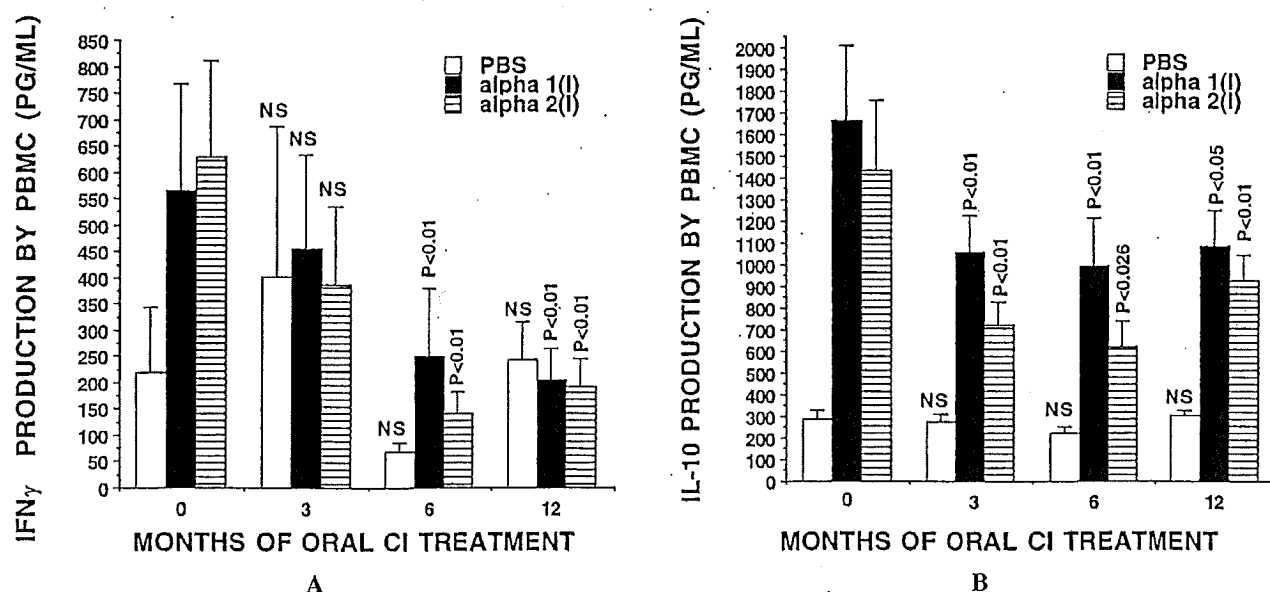


Figure 1. Production of A, interferon- γ (IFN γ) and B, interleukin-10 (IL-10) by peripheral blood mononuclear cells (PBMC) from patients with systemic sclerosis. PBMC were cultured with α 1(I) and α 2(I) chains before and after 3, 6, and 12 months of oral treatment with bovine type I collagen (CI; 500 μ g/day). Harvested culture supernatants were analyzed for levels of IFN γ and IL-10 by commercial enzyme-linked immunosorbent assay, as described in Patients and Methods. Values are the mean and SEM. *P* values determined by Student's paired *t*-test; NS = not significant.

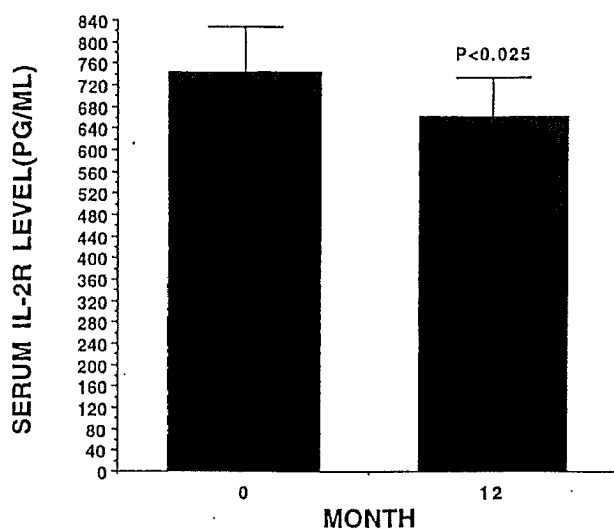
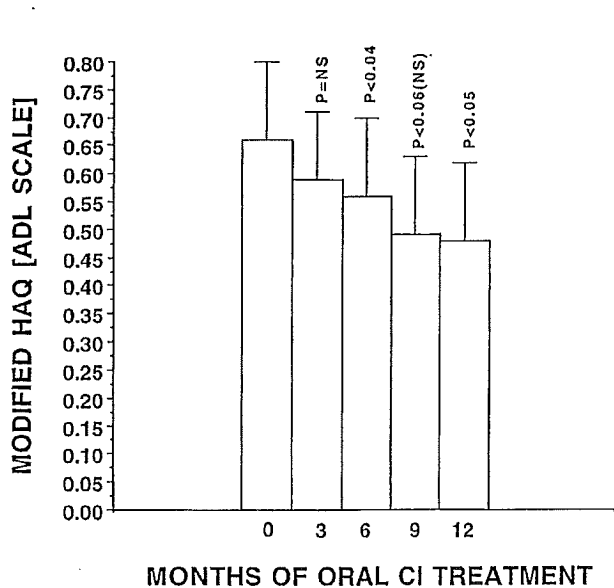


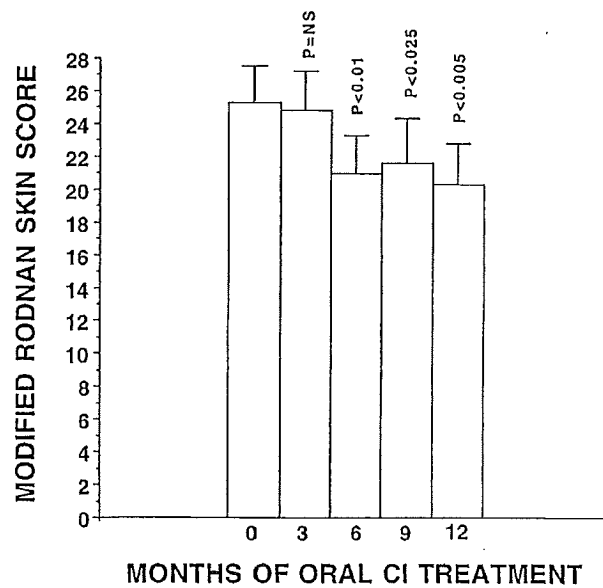
Figure 2. Serum levels of soluble interleukin-2 receptor (IL-2R) in patients with systemic sclerosis. Sera were obtained before and after 12 months of oral treatment with bovine type I collagen and analyzed by commercial enzyme-linked immunosorbent assay for soluble IL-2R levels. Values are the mean and SEM of the 17 patients completing 12 months of treatment. *P* value determined by Student's paired *t*-test.

Improvement in clinical variables. The M-HAQ difficulty in performing activities of daily living (ADL) scale and the MRSS were significantly improved after 6 and 12 months in this open-label study (Figures 3A and B). After 12 months of CI treatment, the M-HAQ ADL difficulty scale had improved 27%, from a baseline value of 0.66 ± 0.14 (mean \pm SEM) to a value of 0.48 ± 0.14 ($P < 0.05$). The MRSS declined steadily, and after 12 months of CI treatment, had decreased by 23%, from a baseline value of 26.35 ± 2.35 to a value of 20.29 ± 2.53 ($P < 0.005$) (Figure 3B). In the patients with diffuse SSc, the MRSS decreased by 26.6% after 12 months of CI treatment, from a baseline value of 28.6 ± 2.5 to a value of 21.0 ± 2.7 ($P < 0.005$) (results not shown). There were no significant correlations between MRSS score or M-HAQ score and decreases in IL-10 or IFN γ production by PBMC cultured with CI α chains after 12 months of CI treatment (results not shown).

Because of patient noncompliance and scheduling problems, only 11 of the 17 patients who finished the study had DLco measurements and PFTs performed at 0 and 12 months. For these 11 patients, the mean DLco, corrected for alveolar volume and hemoglobin, increased by 9.58% from 3.34 to 3.66 ml/minute/mm Hg (*P*



A



B

Figure 3. A, The modified Rodnan skin thickness score (MRSS) and B, the modified Health Assessment Questionnaire (M-HAQ) scores in patients with systemic sclerosis. The MRSS and the M-HAQ were measured before and after 12 months of oral treatment with bovine type I collagen (CI). Values are the mean and SEM. *P* values determined by Student's paired *t*-test; NS = not significant. ADL = activities of daily living.

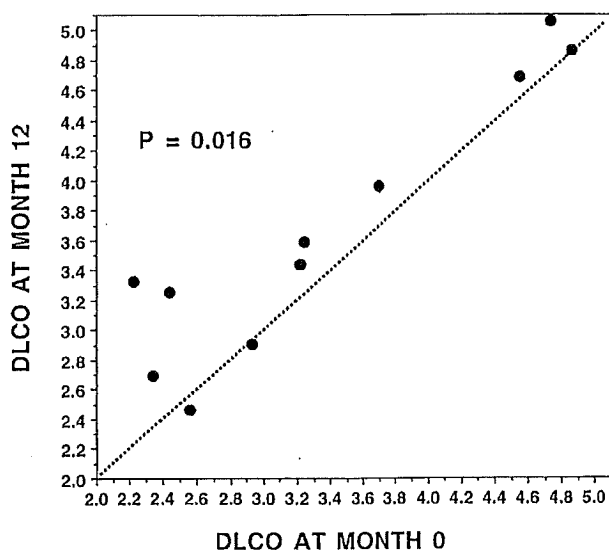


Figure 4. Diffusing capacity for carbon monoxide (DLCO) in 11 patients with systemic sclerosis, before and after 12 months of oral treatment with bovine type I collagen. The dotted line is a reference line with a slope of 1. *P* value determined by Student's paired *t*-test.

< 0.016) after 12 months of CI therapy (Figure 4). There was no significant change in forced vital capacity or in serum creatinine values.

DISCUSSION

In patients with limited and diffuse SSc, the administration of oral bovine CI for 1 month at a dosage of 100 μ g/day and for 11 months at 500 μ g/day resulted in significant reductions in IFN γ and IL-10 production by CI α -chain-stimulated PBMC. Levels of serum sIL-2R were also significantly reduced after induction of oral tolerance to CI. This reduction in sIL-2R, although small, suggests that overall T cell stimulation was reduced. To our knowledge, levels of sIL-2R in sera have not been reported in other studies of human oral tolerance or in animal models of oral tolerance. Therefore, the significance of this modest reduction in serum sIL-2R levels in the context of oral tolerance is not known at present. Taken together, these data indicate that oral tolerance to CI was effected by this treatment regimen.

The mechanism(s) by which this oral CI regimen induced these immune changes is not readily apparent. It is likely that IFN γ production by CI α -chain-stimulated PBMC is largely from CD4 $^{+}$ Th1 cells; but natural killer cells are also a potential source of this

cytokine. The reduced production of IFN γ by PBMC CD4 $^{+}$ T cells could be due to 1 or a combination of the 3 mechanisms of oral tolerance induction (i.e., suppressive regulatory T cells, clonal anergy, or clonal deletion) (10–12).

The reduced IL-10 production by SSc CI α -chain-stimulated PBMC after oral CI treatment was unexpected, given that IL-10 has been reported to be up-regulated in peripheral lymphoid tissue or in target organs in autoimmune immune models after oral tolerance induction by low-dose antigen (20). However, in some circumstances, IL-10 can be produced by Th1 cells, and in humans, there is less rigidity to the Th1/Th2 paradigm originally described using clonal mouse T cells (20–22). In addition, IL-10 is produced by cells other than CD4 $^{+}$ T cells (23,24). Monocyte/macrophages are a major source of this cytokine (23,25). The reduced IL-10 production by CI α -chain-stimulated SSc PBMC after oral CI treatment may reflect overall decreased T cell responsiveness to the α chains, and therefore decreased stimulation to monocytes by IFN γ or other cytokines from T cells that up-regulate IL-10 production by monocytes. Finally, published studies of oral tolerance in animal models have not measured antigen-stimulated PBMC production of IL-10, or other cytokines elaborated by PBMC, before and after oral tolerance induction. The published studies of animal models of oral tolerance all measure cytokine expression in lymphoid tissue or target organs rather than peripheral blood.

NSAIDs are known to inhibit oral tolerance in animal models and may be a confounding factor in human oral tolerance (26–28). For this reason, we advised the 5 patients who had been taking NSAIDs for the first 6 months of the trial to discontinue them for the last 6 months of the trial, which they did.

Although there were significant improvements in the MRSS and M-HAQ scores, these findings should be viewed with caution, since this was an open-label study and these changes may reflect variations or spontaneous changes in the disease or a placebo effect. The DLco values, while showing statistically significant improvement, are still just below the clinically significant cutoff of $\geq 10\%$. Clearly, a larger population of more homogeneous patients with diffuse SSc needs to be evaluated in a randomized, double-blind, placebo-controlled study before it can be categorically concluded that oral CI tolerance induction ameliorates the SSc disease process.

One could speculate that the mechanism by which oral CI might possibly ameliorate SSc could involve anergy and/or suppression depending on the

dose of CI given. Although the 500 $\mu\text{g/day}$ dosage of CI induced oral tolerance, clearly other dosages need to be studied. The feeding of CI to SSc patients could anergize autoreactive cells and/or generate major histocompatibility complex class I- or class II-restricted regulatory T cells that sequester in involved tissues, where they release small amounts of immunosuppressive cytokines (IL-4, IL-10, TGF β 1) that down-regulate autoaggressive cells by the mechanism of antigen-driven bystander suppression. By antigen-driven bystander suppression, these CI-specific T cells could down-regulate T cell interactions with other antigens (29), as has been demonstrated in autoimmune animal models in which oral tolerance has been induced by oral administration of antigens from organs that are the target of attack.

These animal models provide a theoretical basis for predicting that in SSc patients, CI, although it may not be an initiating antigen of SSc or even be involved in its pathogenesis, when given as an oral tolerogen, may well suppress T cell-mediated fibrogenesis by suppressing activated T cells. If activated CD4+ T cells present in the tissues of SSc patients could be down-regulated, then with time, the fibrogenic phenotype of SSc fibroblasts might revert to normal. It is known that after serial passage of SSc fibroblasts in vitro for several generations, they regain a more normal phenotype with regard to matrix synthesis (30). Also, patients with longstanding SSc tend to have less skin thickening and collagen deposition than they had in earlier stages of their disease.

While IL-4 and TGF β at high concentrations (~50 ng/ml and 5 ng/ml, respectively) can up-regulate collagen synthesis by cultured fibroblasts in vitro, lower concentrations (e.g., TGF β 1 at 1,000 times less) are capable of modulating immune cells (30–34). The fact that oral CI treatment did not increase skin or lung fibrosis suggests that if GALT-derived regulatory T cells producing these cytokines were generated by oral CI treatment, the levels of these cytokines are likely to be lower than is required to trigger collagen synthesis by fibroblasts.

Oral CI administration appears to be safe in SSc patients. Its efficacy needs to be assessed by a larger placebo-controlled, double-blind trial.

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Errata

In the article by García-Porrúa et al published in the March 2000 issue of *Arthritis & Rheumatism* (pp 584-592), there was an error in the second full sentence in the left column on page 589. The sentence should have read, "Six of 39 patients diagnosed as having idiopathic EN (15.4%) had 1 or more predictive factors for secondary EN, and 64 of 67 patients diagnosed as having secondary EN (95.5%) had 1 or more predictive factors for secondary EN" [emphasis added]. This is also how the first sentence of the first footnote in Table 4 should have read.

In the article by van der Heijden et al in the March 2000 issue (pp 593-598), the reference cited at the end of the first sentence in the second paragraph of Patients and Methods (page 594) should have been reference 11, rather than reference 4. The reference cited at the end of line 12 in the second paragraph of the Discussion (page 597) should also have been reference 11, rather than reference 8.

We regret the errors.

EXHIBIT 17

LACK OF EFFICACY OF ORAL BOVINE TYPE II COLLAGEN ADDED TO EXISTING THERAPY IN RHEUMATOID ARTHRITIS

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Objective. To investigate the efficacy of oral type II collagen (CII) in the treatment of rheumatoid arthritis (RA), when added to existing therapy.

Methods. Patients with active RA ($n = 190$) were randomized into a 6-month, double-blind, placebo-controlled trial. Patients continued to take their current arthritis medications. Patients received either placebo or bovine CII, 0.1 mg/day for 1 month, then 0.5 mg/day for 5 months.

Results. There were no significant differences between the baseline characteristics of either group. The primary response parameter was the American College of Rheumatology (ACR) preliminary definition of improvement in RA (ACR 20). There was no statistically significant difference in the ACR 20 after 6 months (20.0% of placebo patients; 16.84% of bovine CII patients). There were significant differences in several clinical variables after treatment, all favoring the placebo group.

Conclusion. Oral solubilized bovine CII, added to existing therapy, did not improve disease activity in patients with RA.

In humans the intestines contain the largest collection of lymphoid tissue (~30,000 scattered solitary

lymphoid follicles), collectively called gut-associated lymphoid tissue (GALT) (1). The GALT is particularly adapted to preventing harmful immune responses to the myriad of dietary antigens ingested during a normal lifespan. This hyporesponsiveness to ingested antigens is produced by a phenomenon known as oral tolerance (OT). Oral tolerization, wherein antigen-specific tolerance is induced to autoantigens, has been shown to be efficacious in a number of animal models of autoimmune disease, including type II collagen-induced arthritis (2), experimental autoimmune encephalitis (3), experimental autoimmune uveoretinitis (4), and type 1 diabetes mellitus (5).

Oral type II collagen (CII) has been used to treat rheumatoid arthritis (RA) and juvenile rheumatoid arthritis, with some benefit to patients with these diseases (6-9). In published trials of oral CII treatment of RA, patients have been allowed to continue taking low-dose prednisone and nonsteroidal antiinflammatory drugs (NSAIDs), but not disease-modifying antirheumatic drugs (DMARDs) (6-9). Since RA often worsens when DMARDs are withdrawn, we wanted to determine whether oral CII therapy could be used as a supplemental treatment. Therefore, we conducted a double-blind, placebo-controlled, phase II trial of daily oral bovine CII (0.1 mg/day for 1 month, then 0.5 mg/day for 5 months) in 190 patients with active RA. Patients continued to take their NSAIDs, prednisone (≤ 10 mg/day), and/or DMARDs during the study.

PATIENTS AND METHODS

Patient recruitment and characteristics. Patients were recruited from rheumatology practices at the University of Tennessee, in the community of Memphis, TN, and from newspaper advertisements. Inclusion criteria were as follows: 1) age ≥ 18 years; 2) American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987

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revised criteria for RA; 3) onset of disease at age ≥ 16 ; 4) functional class II or III; 5) active disease, as defined by the presence of ≥ 6 swollen joints and at least 2 of the following: erythrocyte sedimentation rate (ESR) (Westergren method) ≥ 28 mm/hour, morning stiffness ≥ 45 minutes, and ≥ 9 tender joints; 6) if taking concomitant medications, patients must have been on a stable regimen for ≥ 3 months for DMARDs and ≥ 1 month for NSAIDs and corticosteroids. Maximal allowable dosages for concomitant medications were methotrexate 20 mg/week, hydroxychloroquine 400 mg/day, gold sodium thioglucose or gold sodium thiomalate 50 mg every 2 weeks, sulfasalazine 3 gm/day, azathioprine 200 mg/day, auranofin 9 mg/day, D-penicillamine 750 mg/day, and prednisone 10 mg/day. DMARDs could be used in combination.

Patients were excluded from the study if they were unable to render informed consent in accordance with institutional guidelines, had received another investigational drug within 90 days of entry into this study, had used cyclophosphamide or cyclosporin A in the previous 90 days, had a concurrent serious medical condition which, in the opinion of the investigator, made the patient inappropriate for the study, had positive findings on a pregnancy test, or had an allergy to beef.

Design and duration of the study. The study was a randomized, stratified (for DMARD use), controlled, double-blind, phase II trial. Patients received either a daily oral dose of placebo (0.1M acetic acid) for 6 months or 0.1 mg of solubilized bovine CII daily for 1 month, then 0.5 mg daily for 5 months. The collagen was solubilized in 0.1M acetic acid and aliquoted into individual doses. The aliquots were kept refrigerated prior to use and then added to 4–6 ounces of cold orange juice just prior to ingestion.

The study protocol was approved by the University of Tennessee, Memphis, Institutional Review Board.

Concomitant medication. Patients were required to remain on the same stable doses of concomitant DMARD(s), prednisone, and NSAID for the duration of the study. No other analgesics were to be taken within 12 hours of a study visit.

Clinical and laboratory assessments. The following were evaluated or recorded by a blinded examiner at 0, 1, 2, 3, and 6 months: 1) number of swollen joints (of 28 evaluated); 2) number of tender joints (of 28 evaluated); 3) patient's assessment of pain by visual analog scale (VAS); 4) patient's global assessment of disease activity by VAS; 5) physician's global assessment of disease activity by VAS; and 6) patient's assessment of physical function by the modified Health Assessment Questionnaire.

A complete blood cell count, urinalysis, levels of serum electrolytes, glucose, blood urea nitrogen, creatinine, total bilirubin, aspartate aminotransferase, alanine aminotransferase, and ESR were obtained at 0, 3, and 6 months.

To monitor for possible side effects, patients underwent a complete physical examination at 0, 3, 6, and 12 months. Patients who dropped out of the study before completing 6 months of treatment were evaluated fully just before exiting the study.

Criteria for response. The primary response parameter was the ACR preliminary definition of improvement in RA, which requires $\geq 20\%$ improvement in both the swollen joint count and the tender joint count and an additional $\geq 20\%$

improvement in at least 3 of the following 5 parameters: patient's global assessment of disease activity, patient's assessment of pain, patient's assessment of disability, physician's global assessment of disease activity, and acute-phase reactant levels (the ESR) (10).

Preparation and handling of bovine CII. Bovine hyaline cartilage was obtained from twenty 5–7-month-old fetal calves. Knee and hip cartilage was removed by surgical dissection and diced into 5–10-mm cubes. The tissue was homogenized briefly in a Waring blender with ice, then in a tissue homogenizer (model TR10; Tekmar, Cincinnati, OH). Batches of 200 gm were routinely used for each preparation. The procedure for the extraction and purification of CII was previously described (11).

The purified CII precipitate was redissolved in 0.1M acetic acid, dialyzed against 0.005M acetic acid to remove NaCl, aliquoted, and frozen at -80°C until used. The purity of the CII was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, amino acid analysis, carboxymethyl cellulose chromatography of denatured α chains, and by cyanogen bromide (CNBr) digestion of the CII, which yields a collagen type-specific CNBr-peptide map (11).

Frozen CII stock containers were allowed to thaw over 2–3 days at 4°C prior to dispensing into vials. Thawed collagen was centrifuged at 4°C at 12,000g to remove particulates. The collagen was diluted to a concentration of 50 $\mu\text{g}/\text{ml}$ or 250 $\mu\text{g}/\text{ml}$ with cold (4°C) 0.1M acetic acid. The diluted CII was filtered at 4°C through a glass fiber Acrodisc (Gelman Sciences, Ann Arbor, MI) and then a 0.45 μ filter (Nalgene filter; Nalge, Rochester, NY) and aliquoted (2 ml) into sterile screw-top 2-ml polypropylene vials (Nalgene vials; Nalge). Vials ($n = 35$) were placed in plastic bags and stored frozen at -20°C until given to patients.

Placebo (0.1M acetic acid) vials were filtered, aliquoted, and stored in a similar manner. The bovine CII preparation and the placebo had identical appearance, viscosity, and taste.

Statistical analysis. The study had a power of 76% at a 2-tailed level of significance of 0.05 to detect a 20% difference in response rates between collagen and placebo (which was expected to have a 30% response rate). Unpaired *t*-tests were performed on the baseline scores to determine whether there were significant differences between the placebo and collagen groups for 2 samples: the total number of subjects who were enrolled initially, and the 51 subjects who dropped out of the study. Chi-square values were calculated for group status by race and sex for the entire sample, the 51 dropouts, and the remaining subjects.

For the subjects who completed the study, change scores were calculated for each of the independent variables by subtracting the last treatment score from the baseline score. Analysis of covariance was performed on the change scores with baseline as the covariate to determine if there were any differences between the drug and placebo groups. In addition, repeated measures analyses of variance comparing baseline scores with the 4 subsequent treatment scores were performed on the 2 groups to determine if there were any significant differences. Finally, the placebo- and collagen-treated groups were further separated into subgroups, including whether the patient was taking or not taking NSAIDs, DMARDs, or prednisone. Repeated measures analyses of variance compar-

ing baseline scores with the 4 subsequent treatment scores were then performed on the subgroups for each variable to determine if there were any significant differences in the outcome variables. In addition, baseline scores were analyzed separately using a two-way analysis of variance of the collagen and placebo groups with the separated subgroups.

RESULTS

Patient characteristics at study entry. One hundred ninety patients were enrolled in the study (95 in each group), of whom 138 were women and 52 were men. Table 1 summarizes the patients' characteristics at study entry. The groups were similar demographically and in terms of disease characteristics. A significant number of patients in both groups were African American. Disease duration was prolonged in both groups, with means \pm SD of 13.9 ± 8.9 years and 12.8 ± 11.0 years for the placebo- and collagen-treated groups, respectively. Both groups exhibited severe disease, with ~75% being positive for rheumatoid factor and 85% taking DMARDs.

Side effects and withdrawals. There was a high, although not statistically significantly different, dropout rate in both groups: 26 from the placebo group (27.4%),

Table 1. Baseline characteristics by treatment group*

Characteristic	Placebo group (n = 95)	Collagen group (n = 95)
Age, years	56.2 \pm 12.7	53.7 \pm 12.4
Sex, no. of patients		
Male	25	27
Female	70	68
Race, no. of patients		
African American	16	15
White	79	78
Disease duration, years	13.9 \pm 8.9	12.8 \pm 11.0
Rheumatoid factor, % positive	76.8	79.0
NSAID use, %	77.9	73.7
DMARD use, %	85.3	85.3
Swollen joint count (maximum 28)	11.0 \pm 5.4	10.9 \pm 4.8
Tender joint count (maximum 28)	15.5 \pm 7.4	16.5 \pm 6.6
M-HAQ score	23.1 \pm 9.4	23.1 \pm 8.9
Patient's pain assessment, mm (by VAS)	57.6 \pm 20.9	58.2 \pm 23.4
Patient's global assessment, mm (by VAS)	62.7 \pm 20.6	59.8 \pm 22.1
Physician's global assessment, mm (by VAS)	59.4 \pm 17.8	55.5 \pm 18.9
ESR, mm/hour	44.0 \pm 27.2	41.0 \pm 27.7
Morning stiffness, hours	2.6 \pm 1.9	2.8 \pm 3.6

* Differences between groups were not statistically significant. Except where indicated otherwise, values are the mean \pm SD. NSAID = nonsteroidal antiinflammatory drug; DMARD = disease-modifying antirheumatic drug; M-HAQ = modified Health Assessment Questionnaire; VAS = visual analog scale; ESR = erythrocyte sedimentation rate.

Table 2. Side effects and withdrawals from study among the 190 rheumatoid arthritis patients initially randomized*

	Placebo group (n = 95)	Collagen group (n = 95)
Withdrew from study	26	25
Disease worsening	4	10
Protocol violation	10	12
Intercurrent illness	9	3
Possible toxicity	3	0

* Thirteen of the 203 patients who were initially registered were not randomized due to inadequate disease activity.

25 from the bovine CII group (26.3%). Almost all dropouts were due to a lack of efficacy (Table 2). Twenty-two of the 25 dropouts in the collagen-treated group were due to disease worsening or protocol violations. Fourteen of 26 dropouts in the placebo-treated group were due to disease worsening or protocol violations. The protocol violations in both groups consisted almost entirely of increased dosages of antirheumatic drug therapy begun by the patient or the patient's personal physician in response to disease worsening.

CII therapy was remarkably well tolerated. No side effects thought to be probably related to placebo or collagen treatment were noted. Three patients with rashes dropped out because of possible toxicity. These 3 patients all received placebo.

Response. There was no statistically significant difference in response rates (by ACR criteria for improvement in RA) between the placebo (20.0%) and the CII (16.84%) treatment groups. The response rates for those completing the study were also not statistically significantly different: placebo 27.5% (n = 69) and CII 22.89% (n = 70). Analysis of subgroups, including non-DMARD patients and non-NSAID patients, revealed no significant differences in response; however, these subgroups were all numerically small.

When only patients who completed the study were considered, there were significant differences between the placebo and CII groups in some individual clinical variables after 6 months of treatment; these differences all favored the placebo group (Table 3). Placebo-treated patients had significantly more improvement in the physician's global assessment, patient's global assessment, and pain score when compared with patients receiving CII (Table 3). There were no significant differences in swollen or tender joint counts between CII and placebo-treated patients (Table 3).

DISCUSSION

This study shows that daily oral treatment with bovine CII (0.1 mg/day for 1 month, increased to 0.5

Table 3. Differences in individual clinical variables between the placebo and collagen groups*

Variable	Baseline		Study completion	
	Placebo (n = 69)	Collagen (n = 70)	Placebo (n = 69)	Collagen (n = 70)
Swollen joint count (max. 28)	11.0 ± 5.43	10.9 ± 4.76	8.7 ± 4.80	9.1 ± 5.45
Tender joint count (max. 28)	15.5 ± 7.44	16.5 ± 6.57	12.5 ± 7.62	18.5 ± 3.40
Pain, mm (by VAS)	57.6 ± 20.88	58.2 ± 23.39	39.1 ± 26.45	51.1 ± 24.79†
Physician's global assessment, mm (by VAS)	59.4 ± 17.78	55.5 ± 18.90	44.2 ± 20.35	50.0 ± 22.13‡
Patient's global assessment, mm (by VAS)	62.7 ± 20.57	59.8 ± 22.08	43.6 ± 26.02	53.9 ± 26.14†
M-HAQ score	23.1 ± 9.39	23.1 ± 8.88	17.6 ± 10.90	19.1 ± 11.01
ESR, mm/hour	44.0 ± 27.21	41.0 ± 27.74	38.9 ± 26.44	41.3 ± 30.35

* Pretreatment differences between groups were not clinically significant. Differences between groups at study completion were determined by repeated measures of analysis of variance, comparing baseline scores with the 4 subsequent treatment scores. Values are the mean ± SD. VAS = visual analog scale; M-HAQ = modified Health Assessment Questionnaire; ESR = erythrocyte sedimentation rate.

† $P < 0.01$.

‡ $P < 0.05$.

mg/day for 5 months) when added to existing NSAID, prednisone, and/or DMARD therapy in patients with RA does not result in significant clinical improvement in the disease. The possible reasons for this outcome are discussed below.

Results of 3 double-blind, placebo-controlled trials of oral CII therapy in adult RA have been published previously (6,8,9). None of these 3 studies were of identical design, and each was different from the present study. The study by Trentham et al (6) utilized chick CII at a dosage of 100 µg/day for 1 month and then 500 µg/day for 2 months. The 60 RA patients enrolled in that study had a mean disease duration of 10 years, and DMARDs were discontinued the day before oral CII or placebo was started.

In the report by Barnett et al (9), chick CII was also orally administered to RA patients in a multicenter, double-blind, placebo-controlled, randomized trial. A total of 274 patients with active RA and a mean disease duration of 11 years were enrolled in 6 different centers. The patients were randomized to receive placebo or 1 of 4 dosages (20, 100, 500, or 2,500 µg/day) of chick CII for 6 months. There was a variable DMARD washout period prior to entry into the study.

Seiper et al (8) reported the results of a double-blind, placebo-controlled, randomized trial of oral bovine CII in patients with early (≤3 years' duration) RA. Three groups of 30 patients each were randomized to receive placebo or 1 mg or 10 mg of bovine CII daily for 3 months. DMARDs were withdrawn at least 2 weeks before entry into the trial.

The studies by Trentham et al and Barnett et al showed significant improvement in arthritis according to various criteria other than the ACR 20 criteria (6,9,10). In the study by Seiper et al, both the 1-mg/day and the 10-mg/day bovine CII-treated groups showed trends

toward improvement, with the 10-mg/day group showing the stronger trend (8). However, these trends did not reach significance by the ACR 20 criteria.

The major difference between the design of the present study and the 3 earlier trials is that DMARDs, which were taken by 85% of the patients in our study, were not discontinued (6,8,9). There might also be a difference between the tolerogenic properties of chick and bovine CII in humans. While the primary amino acid sequences are highly conserved between chick and bovine CII (~95%, according to GenBank data), a difference in T cell epitope recognition and arthritogenicity has been observed with these 2 species of collagen in B10.RIII mice (12,13). This particular mouse strain develops arthritis when immunized with bovine CII, but not with chick CII (12,13).

The appropriate tolerogenic dose of oral bovine CII that might be optimal in RA is unknown. The study by Seiper et al (8) suggests that a very high dose of 10 mg/day might be better than 1 mg/day. It is known from animal studies that very high oral doses of antigen favor the development of clonal deletion (14), which might maximally benefit RA patients in whom CII might play a significant role in perpetuating the arthritis. Low-dose oral antigen favors the development of regulatory T cells from the GALT that effect "bystander suppression" when they encounter the antigen in the periphery to which they were orally tolerized (15). The dosage of bovine CII that might induce bystander suppression in RA patients might be much lower than the 500 µg/day used in the present study.

It is readily apparent that when used as in the previous 3 studies, neither oral chick CII nor bovine CII is very potent in effecting a reduction in the severity of RA (6,8,9). It is possible that NSAIDs and prednisone used to treat RA, and which have been allowed to be

continued during each of the CII trials reported to date, may interfere with the induction of OT. It has been previously shown in different animal models that NSAIDs abrogate peripheral and oral tolerance induction (16,17). Indomethacin and acetylsalicylic acid administered by intraperitoneal injection were shown by Scheuer et al (16) to block the induction of peripheral tolerance in A/J mice to aggregated human gamma globulin. Louis et al (19) more recently reported that oral administration of indomethacin to BALB/c mice blocked tolerance induction to orally administered ovalbumin. Our own data in CII arthritis-susceptible DBA/1 mice show that orally administered piroxicam or nabumetone blocks the ability of orally administered bovine CII to protect mice against arthritis produced by subsequent immunization with CII (18). Prostaglandins are probably involved in OT induction. We have been able to reverse NSAID inhibition of OT by administering low oral doses of the prostaglandin E_1 analog, misoprostol (19). We have also found that small doses of prednisone (equivalent to 10 mg/day in humans) given orally to DBA/1 mice that were fed CII also abrogate OT induction (Postlethwaite A, Wheller P, Myers L, Kang A: unpublished observations).

Separate and apart from the drugs that RA patients take are possible agents in the foods of the diverse human diet that might interfere with GALT processing of oral antigen. These are not a problem in laboratory animals routinely fed a constant diet of water and laboratory chow. Although oral CII may eventually prove to have a role in the therapy of patients with RA, more basic studies need to be done in laboratory animal models so that protocols can be developed that will optimize the chances of seeing a therapeutic effect.

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EXHIBIT 18

LETTERS

DOI 10.1002/art.20318

Monitoring guidelines for methotrexate-treated rheumatoid arthritis patients: comment on the article by Yazici et al

To the Editor:

In a recent article, Yazici and colleagues used data from a survey of a relatively small number of rheumatologists to support the notion that a laboratory monitoring regimen for methotrexate (MTX)-treated patients with rheumatoid arthritis (RA) that is less intensive than that suggested by the American College of Rheumatology (ACR) is desirable (1). The lack of guidelines for monitoring patients receiving biologic compounds (anti-tumor necrosis factor and anti-interleukin receptor antagonist) was also noted in their report (1). The authors go on to suggest that the guidelines for monitoring MTX therapy need to be updated, and that guidelines for monitoring biologic compounds need to be drawn. Although we strongly agree with the second statement, we feel uncomfortable concluding, based on the data presented, that the ACR guidelines for monitoring MTX therapy need to be modified. Whereas these guidelines are far from being perfect, they are still the best we have; for persons unfamiliar with the original 1994 publication, the guidelines were derived from data based on sound methodology (2).

In a related matter, we want to point out that data gathered in a selected group of patients with RA ($n = 313$), who were followed up at less frequent intervals than those recommended by the ACR, and who only rarely presented abnormal liver function test abnormalities, were presented by Yazici et al at the 2003 annual meeting of the ACR (3). Unfortunately, these data have been quoted on the internet (e.g., eRheumatology News) as coming from the ACR, suggesting that the ACR guidelines are being "adjusted" (4). This, we think, is quite misleading to the clinician given that the ACR has not endorsed the conclusions presented in this abstract.

Is it possible that we have become too complacent in monitoring methotrexate therapy because we may not have personally witnessed a case of clinically significant liver disease? Changing the guidelines should be a meticulously data-driven process rather than based on the current practice patterns of a relatively small group of rheumatologists; at the conclusion of such a process, the guidelines may indeed be changed. If that is the case, we will be the first to welcome such change. Until then, we should use the guidelines as they are.

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DOI 10.1002/art.20572

Reply

To the Editor:

Thank you for the opportunity to respond to the letter by Drs. Alarcón, Kremer, and Weinblatt. With all due respect for the 8 rheumatologists on the Subcommittee on Hepatic Toxicity and MTX of the ACR who developed the current guidelines (1), the number of rheumatologists included in our study ($n = 123$) is 15-fold larger, representing ~5% of practicing rheumatologists in the US. These rheumatologists provided their views concerning standard, everyday care of patients with RA. Furthermore, the current guidelines appear to be based on 446 patients, rather than the quoted 700 (2), 383 of whom had been the subject of 11 previously published studies, 8 of which were continuations of 3 reports of the same patients. This methodology may have led to a misleading total.

I also agree that the current guidelines for MTX monitoring are the best we have, but they are also the only guidelines available. The assumptions concerning clinically significant liver disease rates and the risks associated with liver biopsies (3), as well as the arbitrary choice of testing intervals not supported by data (4), in the development of these guidelines have already been challenged. Other investigators have also suggested modification of the current guidelines, with less frequent laboratory monitoring (5,6). Surely the authors are not suggesting monitoring blood tests every 4 weeks, which would be the literal interpretation of these guidelines.

Drs. Alarcón, Kremer, and Weinblatt also suggest that an abstract presented at the 2003 ACR meeting about the small number of liver function test abnormalities observed among 313 RA patients was presented as an ACR-endorsed statement by a commercial Web site for rheumatologists. This was a newsreel about the abstract about MTX and liver function test abnormalities. There was no indication of an ACR endorsement, and I personally had no control nor any role in the preparation of this newsreel.

Finally, it would be of interest to learn the current prevalence of liver function test abnormalities in patients seen by Drs. Alarcón, Kremer, and Weinblatt, to compare with data reported by me and my colleagues, to help the rheumatology community judge the optimal frequency of laboratory monitoring of patients treated with MTX.

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**HLA-DQA1 as a risk factor for microchimerism:
comment on the article by Artlett et al**

To the Editor:

The recent article by Artlett et al (1) addresses the topic of whether specific HLA alleles are associated with persistent microchimerism, a potential risk factor for autoimmune diseases, as described previously (2). Because the results of the study by Artlett et al are described in comparison with our previous report, I would like to clarify a few descriptions that are not correct. Artlett and colleagues state that, in contrast to our study, they did not find the HLA-DQA1*0501 allele or any other DQA1 allele to be a risk factor for fetal microchimerism in either T lymphocytes or whole peripheral blood from the SSc patient cohort or the controls. First, we never tested whole peripheral blood in our study. Second, although DQA1*0501 was more frequent among women with fetal microchimerism within T lymphocytes, as stated in our article, this finding was not significant when corrected by the number of alleles tested. I am not surprised that Artlett et al did not observe an association with the host's genotype.

In our study, we observed that fetal microchimerism within maternal T lymphocytes was associated with the HLA genotype of the son (donor). Artlett et al describe no significant association of maternal microchimerism within the T lymphocytes of the child according to the presence of DQA1*0501 in the mother ($n = 10$) or of fetal microchimerism within maternal T lymphocytes ($n = 27$) according to the presence of DQA1*0501 in the child. Although the latter result contrasts with our prior report ($n = 29$), it is difficult to draw conclusions for a number of reasons. Perhaps the most important reason is that different techniques were used to detect microchimerism. The study by Artlett et al is heterogeneous for techniques used to detect microchimerism, and it is difficult to ascertain important variables such as the total amount of DNA tested and the purity of sorted subsets. Furthermore, results were combined from quantitative and nonquantitative assays (e.g., a weaker third band was classified as the microchimeric allele), and the criteria used to categorize individuals as positive or negative were not defined. Moreover, quantitative and nonquantitative techniques show different sensitivities, with, respectively, a sensitivity of 1 male cell in 100,000 female cells (3) and 10 cells in 2.4 million host cells (4).

Adding further confusion, either care was not taken in the description of results, or the quantitative results are at marked variance with any other literature report, because in Patients and Methods it is stated that results were normalized to 100,000 autologous cells, and in the Results section ranges of 1-12 (low) and 13-1,130 (high) microchimeric cells are

reported—representing levels as high as 1% of all circulating cells

Methods to detect and accurately quantify microchimerism have advanced considerably since our study in 2000. Further studies will be necessary, in which standardized and carefully conducted techniques are used to study larger numbers of individuals. A final issue we previously raised is whether patients with autoimmune diseases who lack disease-specific HLA molecules could be investigated for persistent microchimerism as an alternative source of HLA disease-associated molecules or peptides, whereas patients who have the disease-specific HLA molecules might not have persistent microchimerism. This is one potential explanation for the observation by Artlett et al that DQA1*0501 was frequently observed in patients who were negative for microchimerism, even though juvenile idiopathic inflammatory myopathy is frequently associated with DQA1*0501.

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Results = 0

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**Dosage effects of orally administered bovine type I
collagen on immune function in patients with systemic
sclerosis**

To the Editor:

Type I collagen (CI), the most abundant protein in humans, may play a pivotal role in the pathogenesis of systemic sclerosis (SSc) (1-6). Oral tolerance studies in rodents have shown that the dose-response curve for orally administered antigen is bimodal, with tolerance being induced optimally using low and high doses of antigen (7). It has been postulated that high-dose oral antigen induces predominantly clonal deletion of antigen-specific T cells, while low-dose oral antigen induces regulatory T cells (8,9). Work from our institution has demonstrated that oral administration of CI at 500 μ g/day in patients with SSc induces tolerance, as characterized by significant reductions in interferon- γ (IFN γ) and interleukin-10 (IL-10) production by peripheral blood mononuclear cells (PBMC) cultured with α 1(I) and α 2(I). This dosage of CI also

effected improvement in several disease parameters, including skin scores (modified Rodnan skin scores [MRSS]) (10), patient assessment of disease activity (modified Health Assessment Questionnaire [M-HAQ]) (11), and results of pulmonary function tests (PFTs), including diffusing capacity for carbon monoxide (DLco) and forced vital capacity (FVC) (12).

Studies in humans with rheumatoid arthritis (9), as well as in animals (13,14), suggest that the oral dosage of an autoantigen such as collagen is pivotal in determining whether tolerance occurs and disease activity is suppressed. In our previous study of oral CI administration to patients with SSc, we used high doses of collagen (500 μ g/day) and did not explore the effects of lower-dose regimens (12). The purpose of the present study was to determine whether lower doses of oral CI (10 μ g/day and 100 μ g/day) would induce immune tolerance to CI in patients with SSc, and/or whether a change in clinical parameters could be effected after 5 months of administration of CI at either or both of these dosages.

The study population consisted of 4 men and 18 women with SSc (17 white, 5 African American). Patients were recruited from the University of Tennessee (UT) and community rheumatology practices in Memphis and surrounding areas. The mean \pm SD duration of disease in the study population was 6.7 ± 7.5 years (range 1 month to 30 years). Eight patients had limited SSc and 14 had diffuse SSc. Written consent for participation in the study was obtained from patients in accordance with the Helsinki II declaration, and the protocol was approved by the UT Institutional Review Board. The study was conducted under US Food and Drug Administration Investigational New Drug application 6575. The inclusion criterion was a clinical diagnosis of limited or diffuse SSc made by the study physician, according to the criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (15). Exclusion criteria were similar to those in our previous study (12); however, treatment with nonsteroidal antiinflammatory drugs (NSAIDs) was not allowed. The rationale for the exclusion of NSAIDs was based on work from our institution suggesting that NSAIDs may impede the development of oral tolerance (16).

For the first 5 months, all patients received placebo (0.1M acetic acid). Patients were then randomized to receive CI at 10 μ g/day or 100 μ g/day for 5 months. Those who had received the 10 μ g/day CI dosage for 5 months were then crossed over to receive the 100 μ g/day dosage for 5 months, and vice versa. Collagen was solubilized in 0.1M acetic acid and aliquoted into individual-dose vials which the patients kept refrigerated. Each morning, the patient added 1 vial of the CI preparation to 4–6 ounces of orange juice, and this was consumed before breakfast. Compliance was monitored by counting the milliliters of study drug left in vials returned at each visit. Patients were considered to have complied with the protocol if they have consumed at least 70% of the study medication. The mean consumption was 88.8% (range 71.2–96.9%).

All assessments were performed at 0, 5, 10, and 15 months. The MRSS (assessed by the same trained physician throughout) and the M-HAQ score were recorded. PFTs, including FVC and DLco studies, were performed by the same technician (under the direction of a pulmonologist), using the same equipment each time. IFN γ and IL-10 levels were measured, by commercial enzyme-linked immunosorbent as-

say (ELISA; R&D Systems, Minneapolis, MN), in supernatants that were harvested from in vitro cultures of SSc PBMCs and stimulated with phytohemagglutinin (PHA; 5 μ g/ml), α 1(I) (50 μ g/ml), or α 2(I) (50 μ g/ml) for 6 days. IFN γ and IL-10 levels in unstimulated PBMC culture supernatants were subtracted at each time point from the levels in supernatants from PBMCs stimulated with PHA, α 1(I) or α 2(I). Serum levels of soluble IL-2 receptor (sIL-2R), a marker of in vivo immune activation, were measured by ELISA (R&D Systems). All samples were tested in duplicate.

Data were analyzed in a covariance structural model to fit a crossover design (PROC MIXED, version 9.1; SAS Institute, Cary, NC). Analyses were performed to determine whether there were significant differences in the mean responses to collagen at 10 μ g/day versus 100 μ g/day, collagen at 10 μ g/day versus placebo, and collagen at 100 μ g/day versus placebo. Results were adjusted for baseline measurements (including MRSS, M-HAQ score, PFT results, and cytokine levels), age, race, sex, disease duration, and type of SSc (limited versus diffuse).

Eighteen patients received 5 months of placebo treatment, 14 received at least 1 dose of oral CI, and 11 completed the entire study. The 11 study withdrawals were due to issues regarding compliance ($n = 6$), traumatic Colles fracture ($n = 1$), intercurrent medical illnesses not related to SSc ($n = 2$), pregnancy ($n = 1$ [during placebo treatment]), and allergic reaction to collagen characterized by a skin rash ($n = 1$). The final sample size was similar to that in our previous study, in which only 17 patients completed the protocol, and of these, only 11 actually had PFTs performed (12). Despite these small numbers, statistically significant results had been demonstrated for immune and clinical parameters, including PFT results, after 1 year of treatment with oral bovine CI at 500 μ g/day (12).

There were no significant differences in response to CI at either 10 μ g/day or 100 μ g/day compared with placebo, with respect to clinical parameters including MRSS, M-HAQ score, and PFT results (FVC and DLco). Interestingly, there was a significant increase in IFN γ production in response to α 2(I) by PBMCs from patients receiving CI at 100 μ g/day compared with both the placebo group ($P = 0.02$) and the 10 μ g/day CI group ($P = 0.01$). There were no significant differences in the IFN γ response to α 1(I) or in the IL-10 response to α 1(I) or α 2(I) among the treatment groups. In addition, levels of sIL-2R did not differ among the groups ($P > 0.05$).

The major finding of our study was that there was no significant response in any of the clinical parameters evaluated, including MRSS, M-HAQ, or PFTs, to oral bovine CI administered at either 10 μ g/day or 100 μ g/day for 5 months. Furthermore, there was no suppression of the T cell response to CI (i.e., reduction in IFN γ and/or IL-10 production) and no change in systemic immune activity. However, with the 100 μ g/day dosage of CI, we did note increased in vitro production of IFN γ ; this response was seen with α 2(I) stimulation, but not α 1(I) stimulation. Increased IFN γ production by peripheral lymphoid cells cultured with tolerizing antigen has been associated with induction of tolerance to some orally administered antigens in humans and mice (17,18). Whether this enhanced IFN γ production in response to α 2(I) represents tolerance induction or whether increased production of endogenous IFN γ is beneficial in SSc requires further investigation. Induc-

tion of IFN γ may be important because of the antifibrotic properties of this cytokine (19).

It should be noted that our results are limited by the small number of patients who completed the study, the wide range of disease duration, and the probable need to administer oral CI for longer than 5 months to observe a change in skin scores or pulmonary function. However, our findings suggest that low-dose CI may have some effect on cytokine profiles in SSC, although no response in any clinical parameters was noted.

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Recommendations regarding individuals in whom bone densitometry should be performed: comment on the article by van Staa et al

To the Editor:

We read with great interest the article by van Staa and coworkers on the bone mineral density (BMD) threshold for prediction of vertebral fractures in patients receiving oral glucocorticoid therapy (1). Compared with non-glucocorticoid users, glucocorticoid users appear to develop fractures at a higher BMD. The American College of Rheumatology recommends therapeutic intervention if the T score for BMD is below -1 in a patient who has had long-term treatment with glucocorticoids (2). The UK National Osteoporosis Society advocates intervention at a T score threshold of -1.5 (3). With the increasing use of bone density testing for diagnosing osteoporosis and establishing fracture risk, inconsistencies have arisen in the way in which bone densitometry is performed and the results interpreted. As an example, T score diagnostic thresholds for postmenopausal Caucasian women not exposed to glucocorticoids may not apply to patients who are receiving glucocorticoid therapy. A similar analogy could be made in applying World Health Organization T score criteria to men and premenopausal women.

To reduce these inconsistencies and improve interpretation and reporting of BMD, the International Society for Clinical Densitometry (ISCD) periodically convenes Position Development Conferences (PDCs). The most recent PDC was held in Cincinnati, Ohio, in July 2003. The ISCD is a not-for-profit multidisciplinary professional society with a mission to

EXHIBIT 19

A pilot study on the immunological effects of oral administration of donor major histocompatibility complex class II peptides in renal transplant recipients

Womer KL, Magee CC, Najafian N, Vella JP, Milford EL, Sayegh MH, Carpenter CB. A pilot study on the immunological effects of oral administration of donor major histocompatibility complex class II peptides in renal transplant recipients.

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Abstract: Oral tolerance is an important physiological mechanism of immune hyporesponsiveness to dietary antigens and the commensal flora of the gastrointestinal tract. Feeding of alloantigens, therefore, has the potential to suppress undesirable immune responses after transplantation. To date, there are no published reports on the effects of such an approach in human transplant recipients. In the present pilot study, we demonstrate complete suppression of baseline indirect alloreactivity in patients with chronic renal allograft dysfunction following the oral feeding of low (0.5 mg/d) but not higher (1.0 and 5.0 mg/d) doses of donor major histocompatibility complex (MHC) class II peptides. The regimen was well tolerated with no evidence for sensitization to the donor antigen. Our results indicate that oral feeding of low dose donor MHC peptide may represent a safe and effective therapy to suppress indirect alloreactivity in renal transplant recipients with chronic allograft dysfunction and warrants further clinical investigation.

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Key words: active suppression – chronic allograft nephropathy – epitope spreading – indirect allorecognition – kidney transplantation – oral tolerance

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Oral tolerance is an important physiological mechanism of immune hyporesponsiveness to dietary antigens and the commensal flora of the gastrointestinal tract. The mechanisms of oral tolerance induction have been studied extensively in murine systems (1, 2) and include anergy (3, 4) or deletion (5, 6) of antigen-specific T cells, immune deviation to a Th2-biased immune response (7) and induction of regulatory Th3 (transforming growth factor β -producing) cells (8, 9). In an effort to exploit these mechanisms as potential clinical therapy, investigators have used oral tolerance strategies with success to inhibit undesirable immune responses in mouse models of human diseases, including multiple sclerosis, rheumatoid arthritis,

inflammatory bowel disease, autoimmune uveitis, type-1 diabetes, and transplant rejection (1). These animal studies paved the way for subsequent clinical trials in autoimmune diseases, which yielded mixed results (9–14). Conceptually, oral immune tolerance might be better suited for solid organ transplantation than for autoimmunity, because the antigens are defined and administration can be timed to the transplant. However, no such clinical trials have been reported in the field of organ transplantation.

The predominant causes of late renal allograft loss/dysfunction are death with a functioning graft, chronic allograft dysfunction and recurrent disease, accounting for nearly 20% of patients on the

kidney transplant waiting list (15). Chronic allograft dysfunction is a poorly defined clinical-pathological entity manifested clinically by a gradual decrease in renal function over months to years after transplantation, coupled with hypertension (HTN) and variable degrees of proteinuria (16). Currently, no effective therapy exists for this condition. It is generally agreed that both alloantigen-dependent and alloantigen-independent factors contribute to the pathophysiology of chronic allograft dysfunction, although these factors need not be considered mutually exclusive (17).

T-cell recognition of alloantigen is the central event responsible for the rejection of allografts. It is generally accepted that there are at least two pathways of allorecognition. In the direct pathway, T cells recognize endogenous major histocompatibility complex (MHC) peptides in the context of intact donor MHC molecules on the surface of donor antigen-presenting cells. With the indirect pathway, donor MHC molecules are shed from the graft and processed by recipient antigen-presenting cells, where they are presented as peptides to T cells in the context of recipient MHC molecules. It has been suggested that the direct pathway is more active early after transplantation, when a large number of donor antigen-presenting cells are present in the allograft. Likewise, the indirect pathway may cause a chronic smoldering type of rejection, as donor MHC molecules are continually shed from the graft.

Animal studies have provided a definitive link between indirect allorecognition and transplant arteriopathy (18, 19), one of the characteristic histopathologic findings on biopsies with chronic allograft dysfunction. Clinical studies have demonstrated that lymphocytes proliferating to synthetic donor MHC peptide (i.e., indirect alloreactivity) are more prevalent in the peripheral blood of human renal transplant recipients with chronic allograft dysfunction (20, 21). Epitope spreading is defined as the diversification of epitope specificity from an initial focused, dominant epitope-specific immune response, directed against a self or foreign protein, to subdominant and/or cryptic epitopes on that protein (intramolecular spreading) or other proteins (intermolecular spreading). This phenomenon has been defined in experimental and natural situations as a consequence of acute or persistent infection and secondary to chronic tissue destruction that occurs during progressive autoimmune disease. After allotransplantation, epitope spreading is defined as new proliferative responses to donor MHC peptide segments previously eliciting no immunoreactivity and loss of responses to previously immunogenic

peptide segments. This phenomenon has been associated with chronic dysfunction not only of renal (20, 22), but also of cardiac (23) and of pulmonary (24) allografts and is felt to be indicative of chronic persistent immune damage and instrumental in the pathogenesis of this condition. The induction of peripheral tolerance via oral feeding of donor MHC peptides, therefore, has the potential to mitigate at least the immunologic component of chronic renal allograft dysfunction (25, 26).

In this pilot study, we examined the effects of oral donor MHC peptide feeding on subsequent *in vitro* T-cell proliferative responses to individual donor MHC peptide segments in a group of renal transplant recipients with chronic allograft dysfunction.

Patients and methods

Patients

All subjects provided written informed consent in accordance with an approved protocol. Renal allograft recipients transplanted at least six months earlier and who were attending the outpatient clinic of Brigham and Women's Hospital were screened. All such patients were maintained on triple-therapy immunosuppression with cyclosporine A, azathioprine and corticosteroids. HLA-DR2 peptide was chosen for oral feeding, as this molecule is the most prevalent MHC class II molecule in the human population and would therefore increase the pool of potential donor kidneys. Thus, patients of specific interest were those recipients mismatched for HLA-DR2 present in the donor. As we have previously demonstrated a high prevalence of indirect alloreactivity in renal allograft recipients with a serum creatinine greater than 2.0 mg/dL, we used these criteria for further screening (20).

HLA peptides

A panel of overlapping peptides corresponding to the full-length β -chain hypervariable regions of HLA-DRB1*1501 (HLA-DR2, residues 6–21, 22–41, 42–62, 63–80, 81–94) was synthesized (Quality Control Biochemicals, Hopkinton, MA, USA), as previously described (20). Peptides were suspended in phosphate-buffered saline at a concentration of 1 mg/mL for later *in vitro* use. For oral feeding, these identical HLA-DRB1*1501 (HLA-DR2) peptide segments were provided by Autoimmune Inc. (Pasadena, CA, USA). Purity of peptide from either source was confirmed at greater than 95%

by high-performance liquid chromatography and mass spectrometry.

Peptide proliferation assays

Peripheral blood mononuclear cells from renal transplant recipients meeting the criteria above were separated by Ficoll-Hypaque density gradient centrifugation, washed twice and resuspended in RPMI 1640 medium containing 10% normal human serum, 10 mM HEPES buffer, and 100 µg/mL penicillin and streptomycin. These cells were co-cultured at 37°C, 5% CO₂ and 100% humidity for seven d with the individual synthetic donor MHC peptides or mumps antigen (positive control) at 50 µg/mL in flat bottom, 96 well plates. Control cells were cultured in medium alone. Individual experiments were performed in quadruplicate. Lymphocyte proliferation was determined by measuring the DNA incorporation of ³H-thymidine added for the final 18 h of culture using a beta scintillation counter (Wallac, Gaithersburg, MD, USA). Background proliferation was assessed after incubating the cells in culture medium without the peptide. The stimulation index (SI) for each peptide segment was calculated according to the formula: experimental counts per minute (CPM)/control CPM (SI > 2 considered positive), as previously described (20). Data are expressed as mean ± SEM.

Oral feeding

Those patients exhibiting significant *ex vivo* indirect alloreactivity to any of the five HLA-DR2 peptide segments based on two or three peptide proliferation assays were eligible for MHC peptide feeding. These individuals were instructed to swallow a pre-measured aqueous gel containing all five HLA-DR2 peptide segments each morning prior to breakfast, followed by a drink of water or orange juice. To avoid any potential interactions, patients were instructed not to eat or take medications for 30 min following this dose. The dose of peptide was escalated in the following fashion: 0.5 mg/d (of each individual peptide segment) for 30 d, 1.0 mg/d for 30 d and 5.0 mg/d for 30 d. Within three d of the end of each 30-d feeding period and prior to the next stage, peptide proliferation assays were performed to monitor for the presence or absence of indirect alloreactivity.

Panel reactive antibody

Serum samples, pre- and immediately after completion of the peptide feeding, were tested for

anti-HLA-DR2 antibodies with an antigen-specific antibody bead assay by flow cytometry to rule out sensitization to the orally fed donor antigen.

Results

Patient recruitment

Four patients were identified who met the criteria outlined above. Mean time after transplantation was 12.75 ± 1.65 months. All four subjects demonstrated significant alloreactivity to at least one synthetic MHC peptide segment on two or three consecutive baseline monthly peptide proliferation assays, as shown in Table 1. Furthermore, all four subjects demonstrated epitope spreading, as evidenced by shifting proliferative responses over time to the different segments of the HLA-DR2 molecule, consistent with our previous report in patients with renal allograft dysfunction (20). In all but two of these assays, significant proliferation to mumps antigen occurred. Synthetic HLA-DR peptide feeding was carried out in all four subjects, with peptide proliferation assays performed after each stage of feeding. Patient 4 was removed from the study during the second month of feeding because of post-transplant complications unrelated to the study treatment. However, peptide proliferation assays for this patient prior to feeding and immediately following feeding of the 0.5 mg dose were completed and included in this report.

Suppression of indirect alloreactivity after low dose HLA-DR2 peptide feeding

As displayed in Table 1, complete suppression of proliferative responses to the entire panel of HLA-DR2 peptide segments occurred after feeding of the lowest dose of MHC peptide (0.5 mg/d). Patients 3 and 4 also demonstrated suppression of responses to mumps antigen in these assays. Proliferation assays following the higher doses of MHC peptide feeding yielded mixed results, with complete suppression of all peptide segments noted only in the final assay of patient 3.

Absence of sensitization following donor MHC peptide feeding

Results from the flow cytometry for detection of antibodies to HLA-DR revealed no sensitization to donor HLA-DR2 antigens following MHC peptide feeding in any of the study subjects. There were also no episodes of acute rejection or major changes in the level of renal function in any of the subjects during the study period.

Table 1. Peptide proliferation assay results

Assay	DR2#1	DR2#2	DR2#3	DR2#4	DR2#5	Mumps
1.						
Pre-feeding #1	9.2 ± 5.9	32.9 ± 1.3	1.4 ± 0.5	13.6 ± 2.8	35.9 ± 9.6	16.7 ± 1.2
Pre-feeding #2	1.2 ± 0.1	4.5 ± 1.6	1.8 ± 0.2	3.3 ± 0.5	6.3 ± 1.9	2.8 ± 0.1
Pre-feeding #3	1.3 ± 0.5	3.1 ± 1.1	0.6 ± 0.2	4.5 ± 0.8	4.2 ± 0.8	3.4 ± 0.4
Post-0.5 mg/d	0.8 ± 0.1	1.2 ± 0.2	0.7 ± 0.1	1.4 ± 0.2	1.8 ± 0.0	3.7 ± 0.8
Post-1.0 mg/d	1.0 ± 0.6	0.5 ± 0.3	3.9 ± 2.0	6.3 ± 1.1	0.4 ± 0.2	2.8 ± 0.2
Post-5.0 mg/d	1.0 ± 0.3	0.6 ± 0.1	0.7 ± 0.1	5.5 ± 0.6	0.4 ± 0.2	6.3 ± 2.7
2.						
Pre-feeding #1	1.4 ± 0.2	1.9 ± 0.3	1.3 ± 0.1	1.8 ± 0.2	2.5 ± 0.2	3.4 ± 0.3
Pre-feeding #2	2.4 ± 0.2	0.6 ± 0.1	1.9 ± 0.1	1.9 ± 0.3	4.7 ± 0.3	0.8 ± 0.4
Pre-feeding #3	1.3 ± 0.2	1.3 ± 0.1	1.3 ± 0.3	1.1 ± 0.1	1.6 ± 0.1	2.1 ± 0.1
Post-0.5 mg/d	1.4 ± 0.2	1.0 ± 0.1	0.9 ± 0.0	0.7 ± 0.1	0.9 ± 0.2	3.0 ± 0.5
Post-1.0 mg/d	3.3 ± 0.5^a	1.1 ± 0.2	3.8 ± 0.3^a	1.6 ± 0.3	0.6 ± 0.1	1.9 ± 0.1
Post-5.0 mg/d	2.8 ± 1.3	2.3 ± 0.2	2.2 ± 0.5	6.2 ± 1.6	4.9 ± 0.6	3.0 ± 0.3
3.						
Pre-feeding #1	1.0 ± 0.1	1.1 ± 0.2	1.3 ± 0.3	1.1 ± 0.1	1.6 ± 0.2	2.3 ± 0.2
Pre-feeding #2	2.3 ± 0.3	0.7 ± 0.2	0.8 ± 0.2	1.0 ± 0.1	1.6 ± 0.1	3.2 ± 0.4
Post-0.5 mg/d	0.8 ± 0.2	1.1 ± 0.2	0.9 ± 0.1	1.1 ± 0.0	0.9 ± 0.2	0.2 ± 0.0
Post-1.0 mg/d	2.4 ± 0.4	4.4 ± 1.7	1.5 ± 0.2	5.2 ± 2.0	6.1 ± 0.9	9.5 ± 0.6
Post-5.0 mg/d	1.3 ± 0.1	1.0 ± 0.2	1.2 ± 0.2	0.8 ± 0.1	0.9 ± 0.3	2.9 ± 0.4
4.						
Pre-feeding #1	1.3 ± 0.1	3.7 ± 0.3	1.4 ± 0.2	7.2 ± 0.6	12.1 ± 0.8	4.9 ± 0.2
Pre-feeding #2	2.4 ± 0.2^a	2.8 ± 0.2^a	2.5 ± 0.2^a	1.1 ± 0.3	2.2 ± 0.2	1.8 ± 0.1^a
Post-0.5 mg/d	1.0 ± 0.1	0.9 ± 0.3	0.8 ± 0.0	1.7 ± 0.2	0.6 ± 0.1	0.5 ± 0.1

Proliferative responses to individual synthetic donor HLA-DRB1*1501 (DR2) major histocompatibility complex segments or mumps antigen by peptide proliferation assay on peripheral blood mononuclear cell samples from the four patients pre- and post-oral feeding of escalating doses of mismatched donor HLA-DR2 antigen.

^aData reported as mean ± SEM.

Bold numbers indicate significant stimulation indices (≥2.0).

Discussion

In this pilot study, we report for the first time the immunological effects of oral donor class II MHC peptide feeding in a small cohort of renal transplant recipients with evidence of indirect alloreactivity and epitope spreading over time, as demonstrated by reactive T cells to the five epitopes of donor mismatched HLA-DR2 in multiple pre-feeding assays. Complete suppression of indirect alloreactivity occurred in all patients after feeding of low dose (0.5 mg/d) but not higher doses (1.0 and 5.0 mg/d) of MHC peptide. Oral tolerance studies in patients with autoimmune diseases have yielded conflicting results with regard to the most effective dose (27). It has been suggested that clonal deletion and/or anergy may result from feeding high doses of antigen, while active suppression/regulation may be a feature of low dose and/or repeated feeding regimens (1). It was beyond the scope of our study to elucidate the mechanisms by which suppression of indirect alloreactivity was achieved, although future trials should include these experiments.

Regulatory cells have the potential to mediate bystander suppression by exerting non-specific

suppressive effects on other antigen-reactive cells in the vicinity, irrespective of their specificity. On the one hand, this phenomenon may allow suppression of multiple alloantigen-specific responses after oral administration of single or limited segments of mismatched donor MHC molecules. On the other hand, this phenomenon could also result in suppression of protective immunity as well. It is possible that the loss of responses to mumps antigen observed in some of the post-feeding proliferation assays is explained by this mechanism. However, suppression of responses to this nominal antigen did not appear to relate to any specific peptide dose and was observed in two patient samples prior to feeding, arguing against bystander suppression. One of the most feared complications of oral administration of antigen is the induction of deleterious immune responses, which has been reported in an animal model of autoimmune diabetes (28). In none of our study subjects did we detect the development of anti-HLA-DR2 antibodies following feeding of this MHC peptide. These findings suggest that oral tolerance strategies can be conducted safely in the renal transplant population.

In conclusion, our results indicate that oral feeding of low dose donor MHC peptide may represent a safe and effective therapy to suppress indirect alloreactivity in renal transplant recipients with chronic allograft dysfunction. Our results need to be confirmed in a larger group of patients over a longer period of time to determine whether these findings are reproducible with other MHC peptides and importantly, sustainable with repeated low dose peptide feeding. Given the large cost of synthesizing MHC peptides, there are practical limitations to this approach. It will therefore be important to determine in future studies whether feeding of the entire length of the HLA-DR2 molecule is required to suppress proliferative responses to the entire MHC molecule or whether feeding of single segments is sufficient, including the ability to suppress responses to other mismatched MHC molecules present in the allograft (i.e., bystander suppression). Whether oral feeding-mediated suppression of indirect alloreactivity will lead to improvement in the long-term survival of renal allografts, as with most proposed therapies for chronic allograft dysfunction, will unfortunately require numbers of patients and length of follow up that are often regarded as cost prohibitive by the pharmaceutical industry.

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EXHIBIT 20

Oral Insulin Therapy to Prevent Progression of Immune-Mediated (Type 1) Diabetes

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ABSTRACT: Repeated ingestion of insulin has been suggested as an immune tolerization therapy to prevent immune-mediated (type 1) diabetes. We performed a placebo-controlled, two-dose, oral insulin tolerance trial in newly diagnosed (<2 years) diabetic patients who had required insulin replacement for less than 4 weeks and were found to have cytoplasmic islet cell autoantibodies (ICAs). No oral hypoglycemic agents were permitted during the trial. Endogenous insulin reserves were estimated at six-month intervals by plasma C-peptide responses to a mixed meal. Positive ICAs were found in 262 (31%) of the 846 patients screened. Of the 197 who agreed to participate, 187 could be followed for 6 to 36 months. Endogenous insulin retention was dependent upon initial stimulated C-peptide response, age at diabetes onset, and numbers of specific islet cell autoantibodies found. Oral insulin improved plasma C-peptide responses in patients diagnosed at ages greater than 20 years, best seen at the low (1 mg/day) over the high (10 mg/day) insulin dose ($P = .003$ and

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$P = .01$, respectively). In patients diagnosed before age 20 years, the 1 mg dose was ineffective, whereas the 10 mg dose actually accelerated C-peptide loss ($P = .003$). There were no adverse effects. If confirmed, these findings suggest that diabetic patients over age 20 years with ICA evidence of late-onset immune-mediated diabetes should be considered for oral insulin at 1 mg/day to better retain endogenous insulin secretion.

KEYWORDS: type 1 diabetes; immune-mediated diabetes; oral insulin tolerance therapy; endogenous insulin reserve; mixed meal tolerance testing; islet cell autoantibodies (ICA); GAD₆₅; IA-2 autoantibodies; insulin autoantibodies; HLA-DR/DQ phenotypes

INTRODUCTION

The immune-mediated form of type 1 diabetes (IMD) is an incurable, life-long disease¹⁻³ replete with life-threatening complications. The Diabetes Control and Complication Trial clearly showed that better diabetes control lowers complication rates,⁴ and good control is more likely when appreciable endogenous insulin secretion is preserved.⁵ Such preserved secretion capacity of pancreatic β cells has been promoted by immunosuppressant agents.^{6,7} The remissions achieved, however, were mostly transient, whereas the possible risks of lymphoma, renal damage, and/or infections likely outweighed the clinical benefits.⁸ The evolution of autoimmunity and insulinitis prior to the onset of clinical diabetes in nonobese diabetic (NOD) mice, as in human diabetes, has enabled investigators to use this animal model to design effective immunotherapies.⁹ On the basis of such animal studies, autoreactivity of IMD can be manipulated by various islet cell autoantigen-based immunotherapies, including systemic or oral administration of antigen to induce either down regulatory processes (e.g., clonal anergy and deletion of autoreactive T cells) or a systemic deviation of autoimmune responses from destructive to nondestructive outcomes (e.g., Th2/3-dominated autoimmune responses with transferable suppression). It is now known that the frequency of diabetes can be lowered by repeated administrations of oral insulin in NOD mice without reducing insulinitis.^{9,10} However, this effect is dose dependent and the benefits seen at 1 mg doses disappear at higher doses (5 mg).^{11,12} On the basis of encouraging results in NOD mice studies, the possible prevention or delay in the onset of type 1 diabetes in high-risk patients was tested by the administration of insulin in the Diabetes Prevention Trial-Type 1 diabetes (DPT-1) and by the administration of nicotinamide in the European Nicotinamide Diabetes Intervention Trial (ENDIT)^{13,14} However, the results were disappointing, in that neither oral insulin at 7.5 mg/day nor low-dose insulin injections were able to prevent disease in either study.^{14,15} In addition, smaller trials performed in Europe also did not show any benefit of oral insulin therapy in the prevention of diabetes.^{16,17} However, these results highlight the need for better methods to detect disease onset, for delineating the critical time to begin intervention, and for determining the effective antigen dosage and time interval for administration. It was, therefore, the aim of this double-blind placebo-controlled study to learn whether oral insulin therapy taken daily from the time of clinical diagnosis of IMD could improve endogenous insulin retention and, if so, at what dose. We report herein that oral insulin delays progression of β cell failure in newly diagnosed patients, but only in those diagnosed after age 20 years who were given low (1 mg) doses.

MATERIALS AND METHODS

Study Subjects

A total of 846 patients were recruited by participating diabetes centers at the following: Children's Hospital of New Orleans and LSUHSC (Drs. Vargas, Chalew, and Maclaren); University of Florida, Gainesville (Drs. Schatz and Maclaren); University of Texas (Southwestern) at Dallas (Dr. Raskin); University of Southern California, Los Angeles (Dr. Zeidler); Joslin Center for Diabetes, Livingston, New Jersey (Dr. Rapaport); Cleveland Clinic (Dr. Rogers); Florida Hospital Diabetes Center, Orlando (Dr. Crockett); Atlanta Diabetes Associates (Drs. Davidson and Bode); Diabetes and Glandular Disease Clinic, San Antonio (Dr. Schwartz); University of Miami School of Medicine (Dr. Marks); Children's Clinic, Tallahassee, Florida (Dr. Deeb); and University of South Florida, Tampa (Dr. Malone and Shah).

Eligibility requirements for ICA screening were diagnosis according to the American Diabetes Association criteria¹ within the preceding two years, and less than four weeks insulin replacement therapy before entering the study. Some patients presented with a type 1 diabetes phenotype (especially children), whereas others did not (especially adults). Those found to have positive islet cell autoantibodies (ICAs) and who agreed to participate were asked to discontinue any oral hypoglycemic agents, and to have their diabetes managed exclusively by diet, exercise, and insulin injections to maintain optimal Hb_{A1C} levels.

Study Design

The study was conducted under an investigator IND No. 48,784 from the Food and Drug Administration, and under informed consent as approved by local institutional review boards. After an Hb_{A1C} test and a mixed-meal tolerance test (MMTT), patients were randomly assigned to placebo, 1 mg, or 10 mg insulin-containing capsules each day, balanced by the study center for age of onset (less than or greater than 20 years and treatment group). Repeat Hb_{A1C} levels were performed, weights recorded, and insulin doses calculated in units/kg/d every three months. At six-month intervals, MMTTs and multiple ICA levels were repeated. Patients were encouraged to remain in the trial for at least one year; for some patients, follow-up was extended up to three years. Patients with minimal plasma C-peptide responses (peak < 0.3 μ U/mL) to the MMTT were considered to be treatment failures, and were removed from further study as identified.

Oral Insulin

The oral insulin used in the study was from a single batch of regular, recombinant human insulin, kindly donated by the Eli Lilly Co., Indianapolis, IN. The Belmar Pharmacy, Denver, CO, produced the insulin capsules under GMP conditions using methyl cellulose as excipient.

Mixed Meal Tolerance Tests

Patients were instructed to take a high-carbohydrate diet for at least three days and then to fast overnight before the test. Sustacal or Boost was given at 7 mL/kg

up to a 13 oz. dose, and blood samples taken for glucose and plasma C-peptide levels at 0, 30, 60, 90, and 120 min thereafter.

Laboratory Analyses

Cytoplasmic islet cell autoantibodies (ICA) were determined using cryocut sections of freshly frozen human blood group O pancreas, as previously described.¹⁸ Results equal to or greater than 20 JDFI units on two separate occasions were considered positive. Insulin autoantibodies (IAA) were assayed by a radio immunoassay binding method using human monospecific A-14A-14 ¹²⁵I-labeled insulin ligand, kindly provided by the Eli Lilly Co. as previously described.¹⁹ A positive result was a displaceable insulin binding of > 105 μ U/mL or mean + 3 SDs of normal controls. Autoantibodies to Mr65Kda human glutamic acid (GAD₆₅A) and to the insulinoma-associated antigen-two (IA-2A) tyrosine phosphatase were performed as previously described by a double radioimmunoassay procedure using tritium and ³⁵S-labeled substrates produced recombinantly in a rabbit reticulocyte expression system.²⁰ Insulin and C-peptide analyses were performed by radioimmunoassay using kits purchased from Diagnostic Products, CA, and Linco, St. Louis MO. The Hb_{A1C} assay used a capillary electrophoretic method.²¹ HLA phenotyping for DRB1 and DQB1 alleles was done by SSP as previously published.²⁰

Statistical Analysis

The study was designed with approximately 60 subjects in each treatment group and had an 80% power to detect a 20% difference in the one-year failure-free survival rate overall and 80% power detect a 25% difference within each stratum. Kaplan-Meier life tables were constructed to describe the time from study enrollment until the peak plasma C-peptide response to MMTT fell below 0.3 μ U/mL, a value that taken as evidence for near complete pancreatic β cell failure. The long-rank test was used to compare the life tables. Multivariate analysis of time until loss of peak C-peptide response to MMTT used the Cox proportional hazards general linear model. Covariates included age, peak C-peptide response, and antibody positivity at trial onset. The loss of C-peptide response over time was analyzed using the repeated measure analysis of variance, incorporating indicator variables for each subject that had serial results. Because the distribution of the C-peptide values were skewed, only log-transformed values were used in our analyses. When frequencies were sufficiently large, the Chi-square statistic was used to compare proportions. Otherwise, an exact test of proportions was used. The three-arm comparisons of variables at selected time points were made using either the Duncan or Tukey multiple-range tests. A two-tail *P* value of .05 was established for statistical significance. Values between .05 and .01 were considered to be marginally significant, to allow for the multiple comparisons made.

RESULTS

The numbers of patients who were screened for ICA, were found to be positive, and who participated in the oral insulin trial, together with their gender, ages, and initial Hb_{A1C} levels, are shown in TABLE 1. There were no statistical differences be-

TABLE 1. Baseline characteristics of the study subjects

	Screened for ICA	ICA+	Completed >6 Months of Trials
Number of Patients	846	262	187
Age Range in Years	5-78	5-72	5-60
Mean Age (years \pm SD)	33.8 \pm 16.1	22.4 \pm 13.8	23.3 \pm 13.7
Male/Female (%)	356/490 (73%)	114/147 (78%)	85/102 (83%)
Initial Hb _{A1C} (Mean % \pm SD)	ND	ND	8.6% \pm 2.4

TABLE 2. Metabolic outcomes and insulin requirements during follow-up

	Patient Treatment Group		
	1 mg Insulin Dose	10 mg Insulin Dose	Placebo
Basal Hb _{A1C} (mean % \pm SD)	8.9 \pm 2.8 (n=62)	8.6 \pm 2.4 (n=62)	8.3 \pm 2.4 (n=63)
6 months Hb _{A1C} (mean % \pm SD)	8.0 \pm 2.1* (n=34)	7.7 \pm 1.6 (n=34)	6.8 \pm 1.4 (n=35)
6 months insulin doses (U/kg/day)	0.46 \pm 0.36 (n=55)	0.43 \pm 0.29 (n=48)	0.43 \pm 0.28 (n=54)
12 months Hb _{A1C} (mean % \pm SD)	8.5 \pm 2.4 (n=27)	7.8 \pm 1.6 (n=20)	8.0 \pm 2.6 (n=22)
12 months insulin doses (U/kg/day)	0.49 \pm 0.33 (n=43)	0.46 \pm 0.31 (n=34)	0.49 \pm 0.31 (n=36)
24 months Hb _{A1C} (mean % \pm SD)	8.5 \pm 3.1 (n=9)	7.8 \pm 0.48 (n=4)	7.3 \pm 1.7 (n=9)
24 months insulin doses (U/kg/day)	0.63 \pm 0.63 (n=14)	0.48 \pm 0.36 (n=12)	0.50 \pm 0.29 (n=15)

*Significantly different from placebo at $P=.03$.

tween the treatment groups for Hb_{A1C} levels or the insulin doses prescribed over the trial (TABLE 2). Subjects were studied for up to three years or until their peak C-peptide responses to a MMTT fell below 0.3 μ U/mL. The median duration of follow-up was one year.

Of 191 randomized subjects, 153 (81%) maintained a C-peptide response greater than the 0.3 μ U/mL threshold. The percentage of the subjects falling below this threshold did not differ significantly between the treatment arms. In a univariate analysis of time until peak C-peptide response failure, initial age and peak C-peptide were both statistically significant ($P=.03$ and $P=.0001$, respectively). Higher baseline C-peptide levels (a measure of endogenous insulin preservation) and older age were both associated with longer times until pancreatic β cell failure (FIG. 1A, B). However, after adjusting the results for the baseline peak C-peptide levels, age at study onset was no longer statistically significant; however, in 38% of subjects under

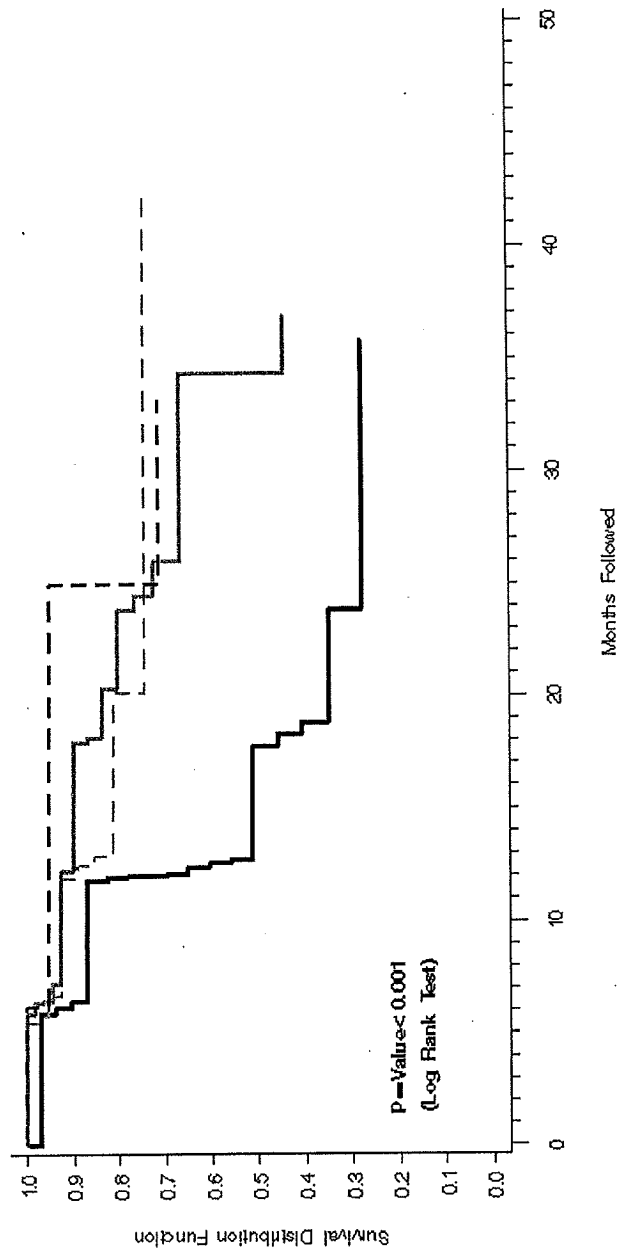


FIGURE 1A. Time until C-peptide loss by age at study onset. *Solid black line, age <10; dashed black line, age ≥20 and <30; solid gray line, age ≥10 and <20; dashed gray line, age ≥30.*

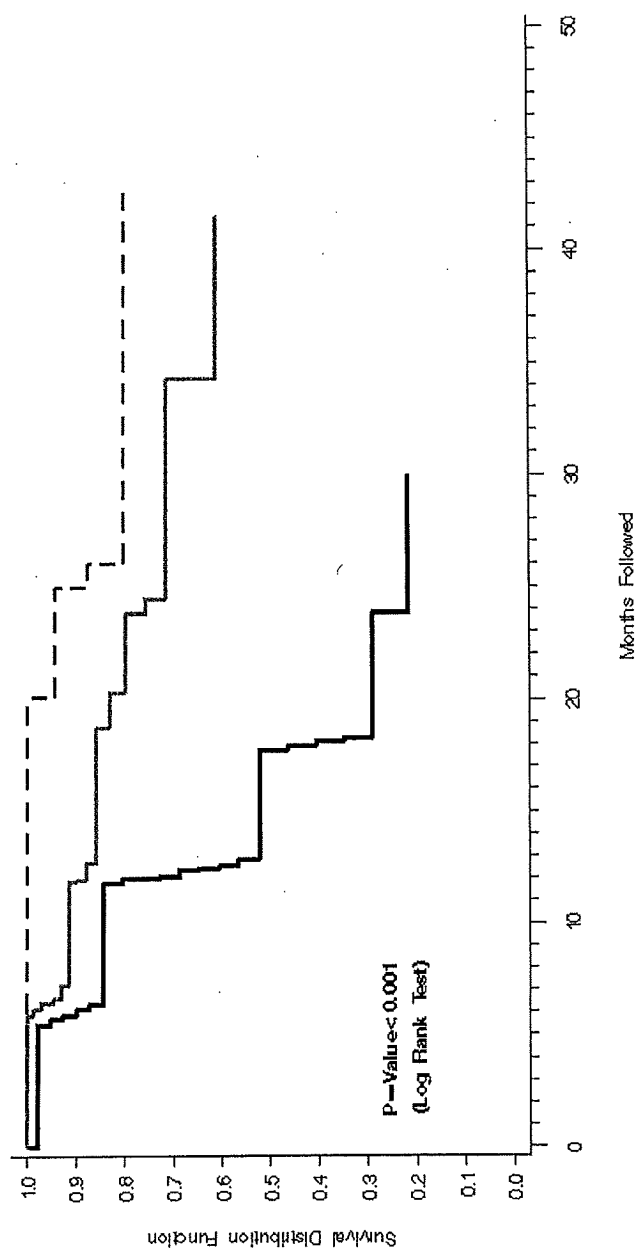


FIGURE 1B. Time until C-peptide loss by baseline C-peptide value. Solid black line, 1.98-3.94; solid gray line, <1.98; dashed black line, >3.94.

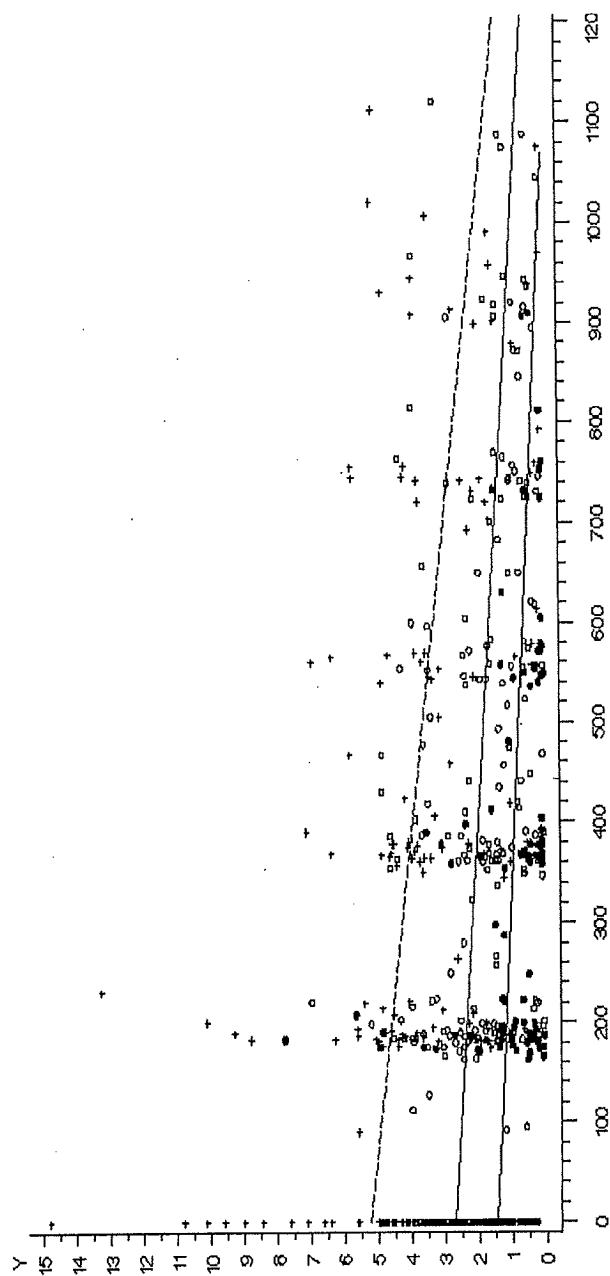


FIGURE 2A. C-peptide values (ng/mL) over time (days) by baseline C-peptide. Solid circles, <1.98 ; open circles, $1.98-3.94$; pluses, >3.94 .

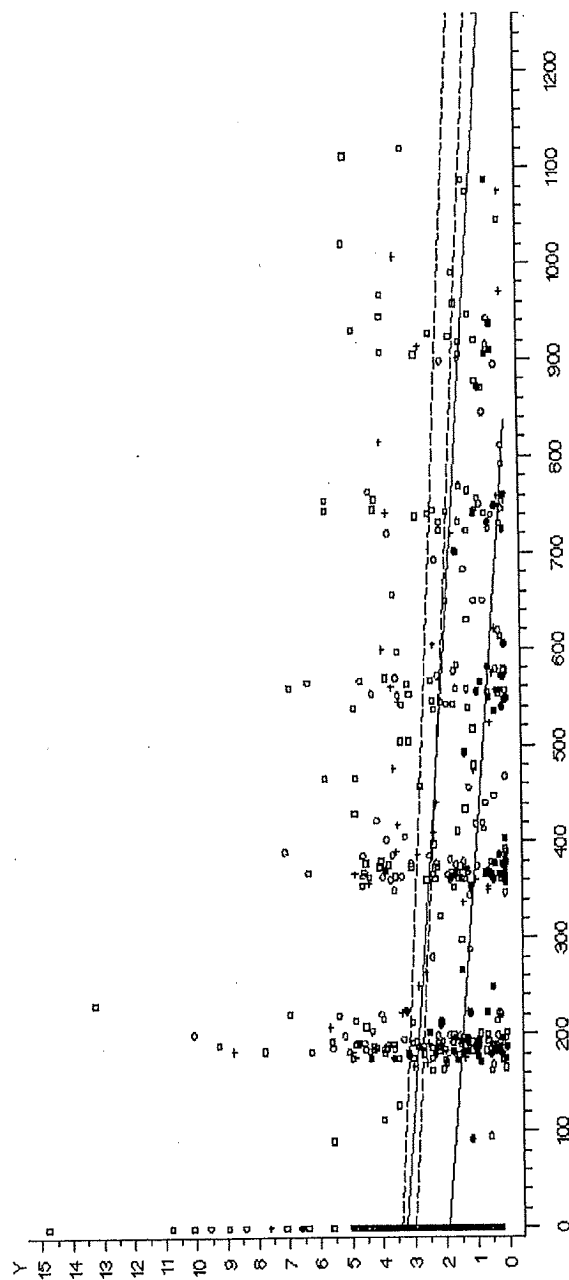


FIGURE 2B. C-peptide values (ng/mL) over time (days) by baseline age. Solid circles, age ≥ 10 and < 20 ; pluses, age ≥ 20 and < 30 ; boxes, age ≥ 30 .

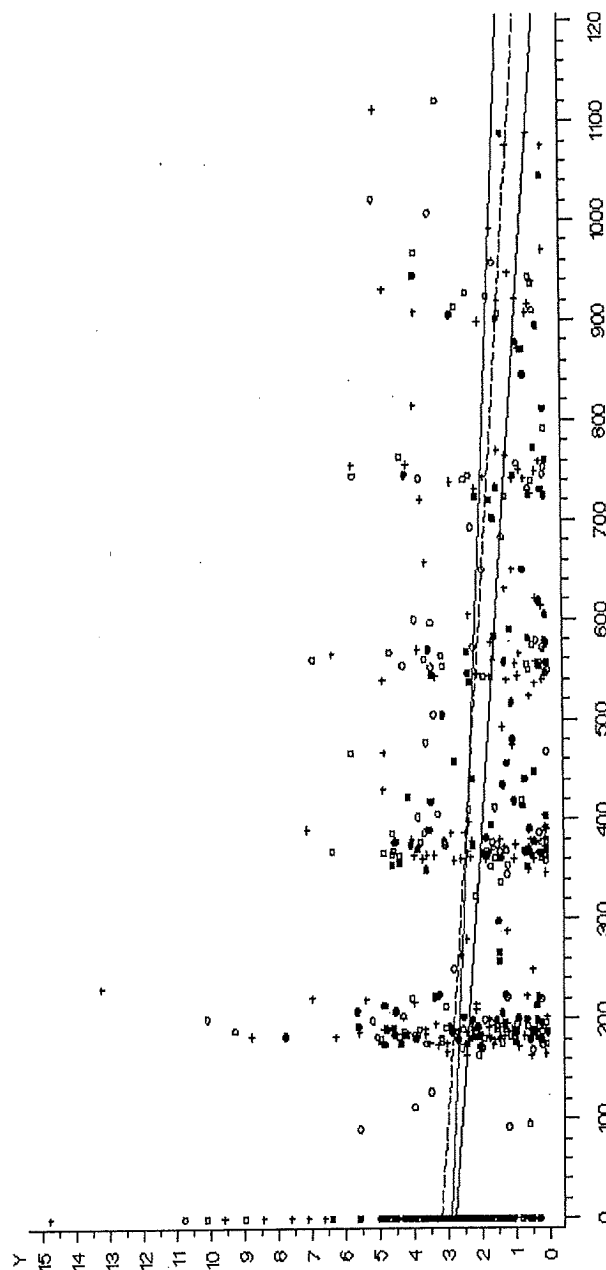


FIGURE 2C. C-peptide values (ng/mL) over time (days) by insulin dose. Solid circles, 10 mg; open circles, 1 mg; pluses, placebo.

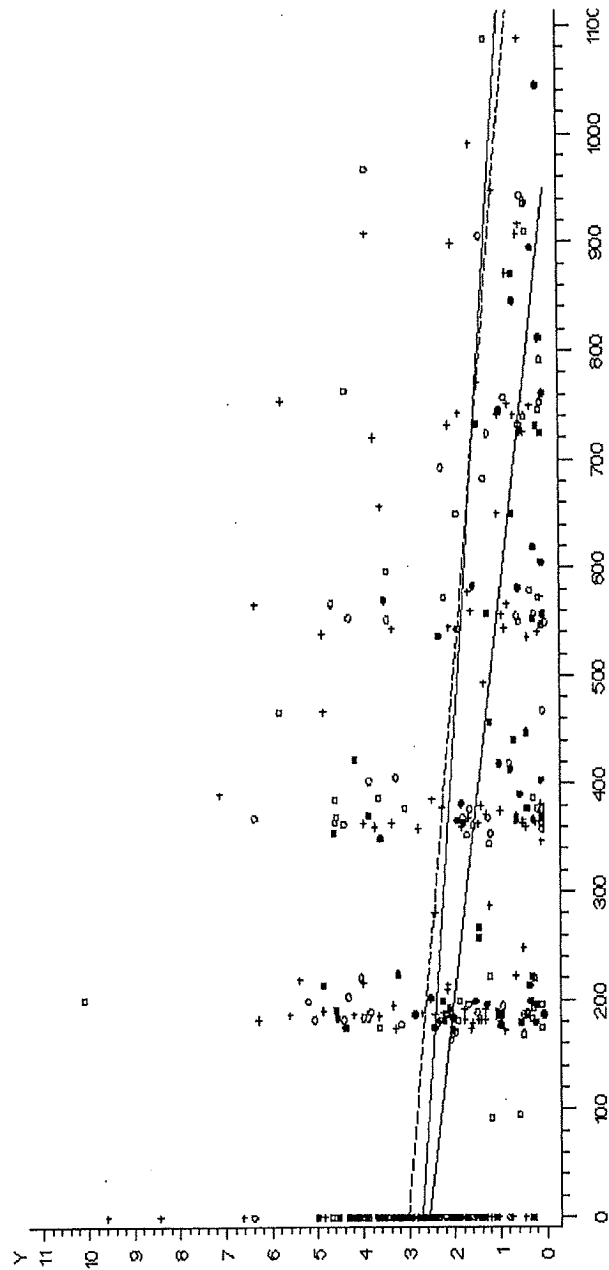


FIGURE 3A. C-peptide values (ng/mL) over time (days) by insulin dose (<20 years of age). Solid circles, 10 mg; open circles, 1 mg; pluses, placebo.

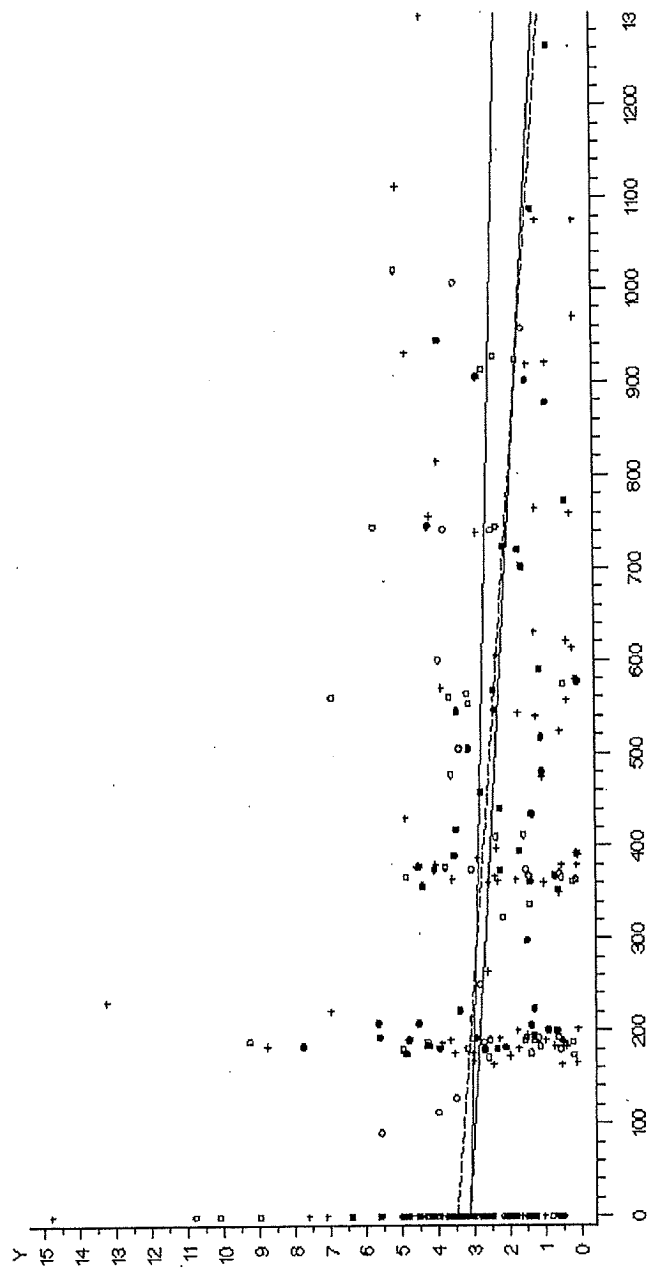


FIGURE 3B. C-peptide values (ng/mL) over time (days) by insulin dose (≥ 20 years of age). Solid circles, 10 mg; open circles, 1 mg; pluses, placebo.

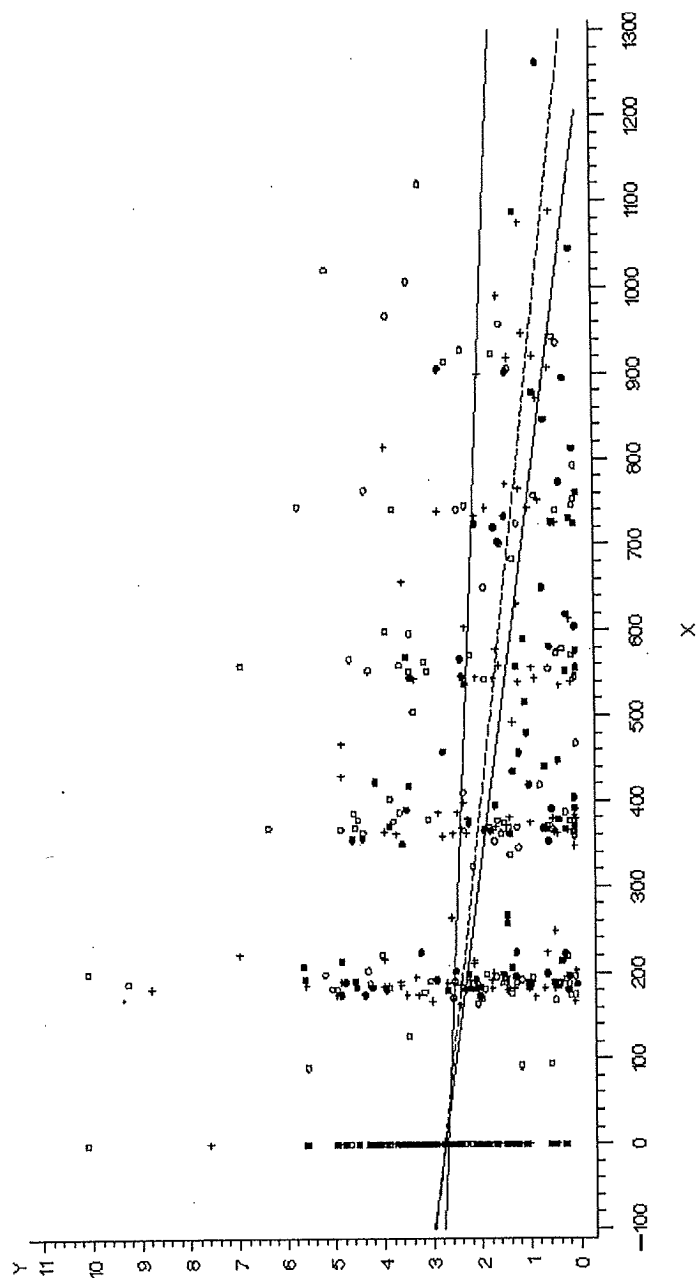


FIGURE 4. C-peptide values (ng/mL) over time (days) by insulin dose (2 or more positive antibodies at study onset). *Solid circles*, 10 mg; *open circles*, 1 mg; *pluses*, placebo.

10 years of age, age and baseline C-peptide levels were correlated with a mean baseline peak C-peptide ≤ 1.98 , as compared with 18, 20, and 24% of subjects in the second, third, or higher decades of life, respectively ($P = .001$). When analyzed by age-at-treatment stratum, baseline peak C-peptide remained a statistically significant prognostic factor in each age group (data not shown).

There were no statistically significant differences in time until failure between the three experimental treatment groups ($P = .42$). The differences in time until C-peptide failure were not statistically significant in either age group (<20 or ≥ 20 years, $P = .08$ and $P = 0.21$, respectively). Although stratified by age, the study was not designed with sufficient power to adequately address this issue.

Changes in peak C-peptide values were also modeled over time to determine whether the rate of C-peptide loss was affected by oral insulin treatment. Whereas the peak C-peptide values generally declined over time, the rate of decline was marginally associated with baseline peak C-peptide levels, with the group with the lowest initial levels having a greater rate of loss after adjusting for repeated observations over time on each subject ($P = .05$; FIG. 2A). The rate of loss in C-peptide was statistically greatest for subjects under 10 years of age at study onset ($P = .0001$) as compared with other age groups (FIG. 2B). Subjects treated with 10 mg of oral insulin had greater declines in peak C-peptide than the others ($P = .02$; FIG. 2C). The greater rate of decline in C-peptide values over time in subjects treated with 10 mg of oral insulin was statistically significant in the group whose baseline C-peptide was between 1.98 and 3.94 ($P = .004$). Within the stratum of subjects under age 20 years at treatment onset, the association between treatment with 10 mg of oral insulin and a greater rate of C-peptide loss was statistically significant ($P = .003$; FIG. 3A). However, among subjects 20 years of age and older, there was a statistically significant benefit of treatment with 1 mg of oral insulin ($P = .002$), even after adjusting for baseline C-peptide values ($P = .003$; FIG. 3B). There was a smaller, yet statistically significant benefit ($P = .01$) from treatment with 10 mg of oral insulin as well in this group.

In a multivariate analysis that included number of specific autoantibodies (ICA, IAA, IA-2A, and GADA) and age at onset, a higher number of autoantibodies was associated with a greater rate of loss of C-peptide ($P = .05$); treatment with 1 mg of oral insulin was significantly associated with a lesser rate of loss of C-peptide in the ICA-only group ($P = .01$), but still statistically significant in those with multiple autoantibodies ($P = .03$; FIG. 4). After adjusting for baseline C-peptide value in the latter group, the lesser rate of C-peptide loss over time with the 1 mg oral insulin dose was still close to statistical significance ($P = .06$).

Patients with the youngest ages of onset had the highest frequencies of at-risk HLA haplotypes and the most numbers of autoantibodies, all of which were associated with the fastest loss of C-peptide response (data not shown).

Overall, we did not observe any side effects from either the low or high oral insulin doses when compared with placebo groups.

DISCUSSION

Oral insulin proved safe without adverse reactions of any kind. Although oral insulin administration did not reduce the overall endogenous insulin failure rate, the

initial C-peptide response, number of specific autoantibodies, and age at onset were variables that were associated with a decreased failure rate. In our study, we demonstrated that in younger patients, more autoantibodies and/or a lower initial C-peptide level were associated with a more rapid loss of peak C-peptide ($\leq 0.3 \mu\text{U/mL}$). However, within the group of subjects with a moderate level of C-peptide at study entry, treatment with the 10 mg dosage was associated with greater rates of C-peptide loss, especially in the group under age 20. In patients over age 20, however, treatment with 1 or 10 mg oral insulin was associated with slower loss of C-peptide response, but not in those whose initial C-peptide responses were very low. Further, the linear dose-response relationship between oral insulin dose and maintenance of C-peptide suggests that there is a therapeutic dose window. Our study indicates that retention of endogenous insulin secretion after diagnosis of type 1 diabetes presenting after the pubertal years can be induced by the sustained, daily ingestion of oral insulin. These findings are in keeping with our preliminary report, which was performed as a blinded, interim safety analysis.²² Further, there was a nonsignificant decline in the level of IAA in patients taking replacement insulin, but no declines in GAD65 or IA-2 autoantibodies. We previously reported that the prevalence of IMD as judged by islet cell autoantibodies and HLA markers of susceptibility for IMD varied among adult diabetic patients according to their ethnicity.²³ If insulin therapy could delay rapid progression to complete β cell destruction in this group, it would be beneficial to their long-term management. The Tokyo study demonstrated that small doses of insulin prevent β cell failure in slowly progressive of type 1 diabetes in adults with high GAD65 antibody levels.²⁴

The lack of adverse effects make this an appealing way to intervene in the autoimmune process that continues beyond the time of clinical diagnosis. As in animal studies, the effect was strikingly dose related, being beneficial at moderate doses but non beneficial or perhaps even harmful at high doses.

The gut-associated lymphoid tissues (GALT) comprise the bulk of the body's immune system, as most foreign antigens are encountered as food. In general, antigen (peptide)-specific regulatory T cells generated by contact with cleaved peptides in the intraepithelial compartment are expanded in local nodes and Peyer's patches, so that subsequent contacts with that antigen will suppress possible T cell activation. Such T cells have a biased expression of cytokines, such as TGF- β , produced by T helper-3 (Th3) cells, or IL-4 and IL-10, produced by Th2 cells. Thus, it was hoped that repeated ingestion of an autoantigen targeted in an autoimmune disease could be used as tolerogenic therapy. Studies in NOD mice indicate that oral feedings of insulin generate insulin/insulin B chain-specific regulatory T cells, which, after clonal expansion, migrate to the pancreatic islets where (insulin/B chain) antigens are found. Once activated, these regulatory T (Tr) cells release their cytokines and thereby inhibit on going *in situ* autoimmune reactions to all antigens,²⁵ a process termed "bystander" immuno suppression. In this study, as in those reported in NOD mice, the protective effect was negated at increased antigen doses.¹¹ Relative to the doses of oral antigen used, the possible outcomes are immunization, active immuno-regulation, or anergy.

Disappointingly, there were no clinical benefits discernible from our oral insulin tolerance therapy as reflected in improved diabetes control, lowered glycated hemoglobin levels, or reduced daily insulin dosages. Because the participating physicians were blinded as to the treatment groups, it is not unexpected that their care would

have achieved similar levels of diabetes control, albeit lower replacement insulin requirements might have been anticipated with improved retention of endogenous insulin from oral insulin therapy, at least in the 20+ years at onset group. Nevertheless, these results are encouraging, and suggest that amendments to the 1 mg dose oral insulin protocol reported herein could lead to improved outcomes, which might be clinically valuable, at least in late-onset disease. Methods of enhancing the beneficial effects could include the addition of a second self-antigen to augment the affect of insulin. Our own group has, in fact, demonstrated augmented effects by the addition of GAD₆₅ to oral insulin in NOD mice.²⁶ Other approaches include adding an adjuvant such as endotoxin²⁷ to promote Th2/3 responses, conjugating the antigen to a hapten such as cholera B chain, and/or adding cytokines therapies such as IL-4 of the Th2/3 types.

Before announcement of the DPT-1 trial,¹⁵ two other trials of oral insulin therapy in newly diagnosed patients with type 1 diabetes were reported without significant improvements in residual β cell function.^{16,17} All three studies used the same type and source of insulin, but the Italian group used a 5 mg dose, the French group 2.5 and 7.5 mg doses, and the DPT-1 trial a 7.5 mg dose. In our study, the 1 mg dose was superior to placebo, but only in patients diagnosed after age 20 years. The above the clinical trials were more limited studies than ours, with generally younger patients presenting clinically with a type 1 diabetic phenotype. The mean age for the Italian study was 14 years, with no patient over 35 years, whereas the mean age of the French study was 18 to 19 years, with no patient over 40 years. In the DPT-1 trial, the median age was 10 years, with a range of 3 to 45 years. This may have been significant, in that we documented worsening C-peptide responses in children, at least at the higher dose used. Further, our study was designed to include many ICA-positive adult diabetic patients in a relatively quiescent phase of their disease. In addition, endogenous insulin reserves in the other two studies were measured after a glucagon stimulus; however, it is unclear whether the mixed meal stimulus we used is the more powerful secretagogue.

Recently published data showed similar results with previous trials in attempt to prevent or delay the onset of IMD; however, preservation of C-peptide production in the prediabetic period appeared to indicate nonprogression to clinical disease.²⁸ This finding may serve as a new surrogate for determining response to preventative efforts.

In summary, ours is the first report to suggest a benefit from oral insulin tolerance therapy in newly diagnosed type 1 diabetic patients, albeit this benefit was chemical rather than clinical, and was confined to patients diagnosed after age 20 years and to low 1 mg daily doses of oral insulin. The result should encourage further trials, with multiple autoantigens (e.g., recombinant human GAD65 and insulin, which give additive protection in NOD mice), adjuvants (e.g., cholera B toxin), and suppressor cytokines (e.g., IL-4, TGF- β , IL-10) to amplify the benefits of oral insulin tolerance therapy, especially in adult-onset patients who have not yet progressed to a type 1 diabetic phenotype.

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EXHIBIT 21

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US91/08143 (22) International Filing Date: 31 October 1991 (31.10.91) (30) Priority data: 607,826 31 October 1990 (31.10.90) US (71) Applicant: AUTOIMMUNE, INC. [US/US]; Reservoir Office Park, 822 Boylston Street, Chestnut Hill, MA 02115 (US). (72) Inventors: WEINER, Howard, L. ; 114 Somerset Road, Brookline, MA 02146 (US). HAFLER, David, Allen ; 110 Forest Avenue, West Newton, MA 02165 (US). CARPENTER, Charles, B. ; 242 Glen Road, Weston, MA 02193 (US). SAYEGH, Mohamed ; 18 Medfield Street, Brookline, MA 02215 (US). ZHANG, Zhengyi ; 130 Avon Street, Malden, MA 02148 (US).		(74) Agents: FRANKFORT, Howard, M. et al.; Darby & Darby, 805 Third Avenue, New York, NY 10022 (US). (81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK, DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent). Published <i>With international search report.</i>
(54) Title: METHODS AND COMPOSITIONS FOR SUPPRESSING ALLOGRAFT REJECTION IN MAMMALS (57) Abstract Disclosed herein are methods for suppressing allograft rejection in mammals comprising administering to a mammal about to undergo or having undergone allograft surgery an agent selected from the group consisting of splenic tissue from an allograft donor, splenic extracts, cultured lymphocytes from an allograft donor, extracts of said cultured lymphocytes, MHC antigens, transplantation rejection suppressive fragments and analogs of MHC antigens in an oral or aerosol form. Also disclosed herein are pharmaceutical formulations and dosage forms for use in said methods.		

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⁺ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

**METHODS AND COMPOSITIONS FOR SUPPRESSING
ALLOGRAFT REJECTION IN MAMMALS**

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FIELD OF THE INVENTION

This invention relates to methods and compositions for suppressing the immune response in animals. More particularly, but not by way of limitation, the present invention is directed to pharmaceutical formulations and methods for suppressing and controlling the immune response of mammals against the introduction of foreign tissue. The invention also includes methods for prolonging the survival of transplanted organs and tissues.

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BACKGROUND OF THE INVENTION

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The success of surgical transplantation of organs and tissue is largely dependent on the ability of the clinician to modulate the immune response of the transplant recipient. Specifically the immunological response directed against the transplanted foreign tissue must be controlled if the tissue is to survive and function. Currently, skin, kidney, liver, pancreas and heart are the major organs or tissues with which allogeneic transplantations are performed. It has long been known that the normally functioning immune system of the transplant recipient recognizes the transplanted organ as "non-

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self" tissue and thereafter mounts an immune response to the presence of the transplanted organ. Left unchecked, the immune response will generate a plurality of cells and proteins that will ultimately result in the loss of biological functioning or the death of the transplanted organ.

Tissue and organ transplant recipients are customarily treated with one or more cytotoxic agents in an effort to suppress the transplant recipient's immune response against the transplanted organ or tissue. For example, cyclosporine (cyclosporin A), a cyclic polypeptide consisting of 11 amino acid residues and produced by the fungus species *Tolypocladium inflatum* Gams, is currently the drug of choice for administration to the recipients of allogeneic kidney, liver, pancreas and heart (i.e., wherein donor and recipient are of the same species of mammals) transplants. However, administration of cyclosporine is not without drawbacks as the drug can cause kidney and liver toxicity as well as hypertension. Moreover, use of cyclosporine can lead to malignancies (such as lymphoma) and lead to opportunistic infection due to the "global" nature of the immunosuppression it induces in patients receiving long term treatment with the drug, i.e., the hosts normal protective immune response to pathogenic microorganisms is downregulated thereby increasing the risk of infections caused by these agents.

Preliminary results have shown FK-506 (which has a similar mode of action as cyclosporine) to be as potent as cyclosporine in its immunosuppressive qualities and to have fewer toxic side effects than cyclosporine. However, because

studies on FK-506 are only in the early stages, it is not available to the general population. Hence, the use of this agent is limited.

Other drugs and/or therapies which are currently administered (either in conjunction with cyclosporine or alone) to suppress the rejection of allogeneic grafts or allografts are also non-specific immunosuppressive drugs or therapies. Steroids, such as prednisone and methylprednisalone, and Azathioprine (an analog of 6-mercaptopurine) are among the non-specific immunosuppressive drugs used to prolong allograft survival in transplantation recipients.

OKT3 monoclonal antibodies, directed against the CD3 antigen present on T-cells, have also been employed as non-specific immunosuppressive therapeutic agents in allograft recipients. However, OKT3 monoclonal antibodies are of murine origin and the patients to whom such monoclonal antibodies are given mount an immune response against these foreign proteins. Thus the usefulness of such materials is limited.

Another drawback to the above-mentioned drugs and antibodies is that they must be administered indefinitely to suppress allogeneic graft rejection, and tolerance to the foreign tissue does not develop.

Total lymphoid irradiation (TLI) is yet another form of non-specific immunosuppressive therapy that has been used clinically and experimentally to prolong allograft survival. The radiation exposure and treatment schedule for TLI were developed for the treatment of Hodgkin's disease and were subsequently found to be immunosuppressive. Although, TLI

induces production of the "global" immunosuppression mentioned above and has the same limitations of other global immunosuppressive therapies, it is the only form of immunosuppression currently in use which appears to induce a specific tolerance to allogeneic tissue. However, TLI is cumbersome to administer and is in an early stage of development, and thus its usefulness is limited.

The oral and aerosol administration of antigens has also been recognized as an effective way to suppress the immune response in mammals to these antigens. The advantages of administering antigens via the oral route include: the simplicity of the techniques involved; the convenience of such techniques since many of the methods can be developed in-situ at the research or treatment facility; the safe, non-toxic effects of the ingestion route; and the specificity that can be provided with the antigens.

Recent studies on several autoimmune disease models have demonstrated that the oral administration of antigens can suppress at least the portion of the immune response that is directed against autoantigens and also protect the treated animals from the induction of specific autoimmune diseases. For example, various animal models are available for the study of Type 1 diabetes as an autoimmune disorder. These include the BB rat (Nakbookda, A.F., et al., Diabetologic 14: 199-207, 1978) and the NOD (non-obese diabetic) mouse in which diabetes develops spontaneously (Prochazka et al. Science 237:286, 1987). Islet-cell specific, CD4- and CD8-positive T-lymphocytes have been implicated as the causative agents respon-

sible for damage to islet beta cells, as demonstrated by transfer of lymphocytes from affected adults to newborn animals (J. Exp. Med. 166:823, 1987).

Experimental allergic encephalomyelitis (EAE) is an induced T-cell mediated autoimmune disease directed against myelin basic protein (MBP) that is widely used as an animal model for the human disease Multiple Sclerosis (MS). EAE can be induced in small mammals by intravenous administration of MBP and a strong adjuvant, such as Freund's complete adjuvant. This treatment induces an acute, monophasic autoimmune disease with the characteristics of MS.

Weiner et al., U.S. Patent Application entitled Method Of Treating Or Preventing Type 1 Diabetes By Oral Administration Of Insulin, filed October 10, 1990, discloses oral and aerosol compositions and pharmaceutical formulations containing insulin which are useful for treating mammals suffering from or at risk for autoimmune diseases having the characteristics of Type 1 diabetes.

Weiner et al., U.S. Patent Application Ser. No. 460,852 filed February 21, 1990, (the national stage of PCT Application No. PCT/US88/02139, filed June 24, 1988), which is a continuation-in-part application of U.S. Patent Application Ser. No. 065,734 filed June 24, 1987, generally discloses the treatment of autoimmune diseases by oral administration of autoantigens.

Weiner et al., U.S. Patent Application Ser. No. 454,486 filed December 20, 1989, discloses the aerosol administration of autoantigens, disease-suppressive fragments of said

autoantigens and analogs thereof as an effective method for treating T-cell mediated autoimmune diseases.

Weiner et al., U.S. Patent Application Ser. No. 487,732, filed March 2, 1990, discloses synergists (enhancers) for use with oral administration of autoantigens, disease suppressive fragments and analogs thereof as effective treatments for T-cell mediated autoimmune diseases.

Weiner et al., U.S. Patent Application Ser. No. 551,632 filed July 10, 1990, a continuation-in-part of U.S. Patent Application Serial No. 379,778, filed July 14, 1989, discloses methods of preventing or treating uveoretinitis in mammals by oral administration of purified S antigen, Interphotoreceptor Retinoid Binding Protein (IRBP) antigen or disease suppressive fragments thereof.

Nagler-Anderson, et al., (Proc. Natl. Acad. Sci. (USA) 83: 7443-7446, 1986), describe the oral administration of collagen to suppress collagen-induced arthritis in a mouse model.

However, the above-mentioned references do not disclose the use of antigens to suppress the mammalian graft rejection mechanism because it has not been shown that the principle of oral administration of transplantation antigens could prevent allograft rejection.

The present invention proposes the clinical administration to mammalian graft recipients of alloantigens via oral and aerosol routes to induce a tolerance to foreign tissue grafts. The invention will be primarily useful in the field of organ transplantation including bone marrow. Although previous

studies have shown that alloantigens injected intravenously to recipients can prolong the survival of renal transplants (Transplantation 39:56, 1985; J. Immunol. 121:1480, 1978; J. Exp. Med. 149:1042, 1979), no disclosure or suggestion of
5 introducing these antigens orally or in an aerosol form was made therein.

It is, therefore, an object of the present invention to provide agents and methods for suppressing the detrimental immune response in mammals to the grafting or transplantation of
10 foreign (or "non-self") tissues and organs.

Another object of the present invention is to provide pharmaceutical formulations and preparations that may be administered to mammals to suppress the immune rejection of surgically transplanted tissues.

15 A still further object of the invention is to provide synthetic compositions and pharmaceutical formulations that may be administered to mammals via the oral or aerosol route to suppress the mammalian immune response to the presence of transplanted tissue or organs.

20 These and other objects of the present invention will become apparent to those of ordinary skill in the art in light of the following.

SUMMARY OF THE INVENTION

It has now been unexpectedly discovered that compositions comprising specific antigenic agents, including by way of
25 non-limiting example allogeneic spleen tissue and cultured lymphocytes and specific Major Histocompatibility Complex (MHC) antigens can be administered to mammals via the oral or aerosol

route to suppress the mammalian immune response to surgically transplanted "non-self" organs or tissues. Because the effect is dependent upon MHC molecules present on the surface of spleen cells, which differ between the tissue donor and the recipient, administration of these antigens alone is expected to be effective.

Orally administered allogeneic splenocytes can suppress the immune response of a host mammal which normally occurs shortly after transplant surgery against surgically transplanted "non-self" tissue in an antigen-specific manner. It has also been found that oral ingestion of allogeneic spleen tissue preparations depresses the delayed type hypersensitivity reaction and mixed lymphocyte reaction in mammals. Compositions and pharmaceutical formulations for oral administration of allogeneic splenocytes may be prepared from natural allogeneic tissue. For administration to humans such compositions comprise synthetic derivatives of antigens i.e., peptide fragments of MHC antigens.

In practicing the method of the present invention, pharmaceutical formulations containing synthetic antigens or natural allogeneic splenic or lymphocyte tissue or cell derivatives are prepared and orally administered to mammalian subjects some time prior to organ or tissue transplant surgery.

Additionally, an aerosol delivery system can be prepared with essentially the dosages of splenocyte derivatives or MHC antigens as above and a pharmaceutically suitable carrier or diluent. The aerosol formulations can also be administered sometime prior to transplant surgery via the

aerosol route. These and other improvements will be described in the following descriptions, drawings and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the effect of feeding
5 allogeneic splenocytes on the mixed lymphocyte reaction (MLR).

Figure 2 is a graph showing the effect of feeding
allogeneic splenocyte lysates on the mixed lymphocyte reaction.

Figure 3 is a graph showing the kinetics of oral
tolerance to alloantigens.

10 Figure 4 is a graph depicting the effect of feeding
syngeneic or allogeneic splenocytes on delayed type hypersensitivity (DTH) reactions.

Figure 5 are a series of immunofluorescence analyses
(histograms) showing the effect of feeding allogeneic
15 splenocytes on lymphocyte composition.

Figure 6 is a graph showing the survival of cardiac
allografts in control rats, LEW rats fed syngeneic splenocytes,
LEW rats fed third party (WF) splenocytes or LEW rats fed
allogeneic splenocytes.

20 Figure 7 is a graph showing the effects of feeding
splenocytes on the MLR of skin graft recipients compared to
control (non-fed) skin graft recipients.

DETAILED DESCRIPTION OF THE INVENTION

25 The contents of all patent applications, patents and
literature references referred to in this specification are
hereby incorporated by reference in their entirety.

The present invention addresses the need for an alternate to existing methods for suppressing the immune response directed against foreign tissue transplants, as for example, post-transplant surgery. In addition, the methods of the present invention provide for prolonged survival of organ and tissue allogeneic grafts (i.e. transplants from individuals of the same species) in a mammal in need of such treatment.

Thus, the present invention provides means whereby the rejection of tissue allografts can be prevented, thus prolonging the survival of transplanted tissue and organs.

It has now been unexpectedly discovered that oral administration of allogeneic splenocytes or synthetic MHC antigens (or immune suppressive fragments or analogs thereof) is effective for suppressing the in vitro mixed lymphocyte reaction which is a model system for the graft rejection response in post-transplant mammalian recipients.

Without wishing to be bound to any particular theory of operation or mechanism of action for the invention it is believed that the oral administration of allogeneic splenocytes or derivatives of MHC antigens pursuant to the present invention affects the immunological mechanisms of graft rejection, i.e. the activation of helper T-cells is decreased by the induction of specific suppressor T-cells.

In the following discussions the following terms shall have the meaning ascribed to them below.

"Oral administration" shall mean both oral administration and enteral administration (delivery directly into the stomach).

"Mammal" shall mean any organism having an immune system and therefore susceptible to allogeneic graft rejection.

"Aerosol" refers to finely divided solid or liquid particles that may be created using a pressurized system such as a nebulizer. The liquid or solid source material contains MHC antigens and/or disease suppressive fragments and analogs thereof as defined herein.

The "aerosol route" of administration means delivery of an aerosol formulation to a host via the nasal or oral airway.

"Major Histocompatibility Complex" (MHC) is defined as a complex series of mammalian cell surface proteins. The MHC plays a central role in many aspects of immunity both in presenting histocompatibility (or transplantation) antigens and in regulating the immune response against conventional (foreign) antigens. There are two types of MHC protein molecules, Class I and Class II. Class I MHC proteins are present on virtually all tissues and Class II MHC proteins are present on the surface of activated T-cells, macrophages and other immune system cells. The human MHC genes (the HLA genetic locus) are located on human chromosome 6, the mouse MHC genes are located in the H-2 genetic locus on mouse chromosome 17 the analogous rat MHC genes are referred to as RTI.

"Class I MHC antigens" are defined as membrane glycoproteins present on the surface of all nucleated cells and play a key role in antigen recognition by CD8+ cytotoxic T-cells.

"Class II MHC molecules" are membrane glycoproteins that form part of the MHC and are most important in the

initiation of immune responses. Class II MHC molecules are found mainly on cells of the immune system including B-cells, macrophages, brain astrocytes, epidermal Langerhan's cells, dendritic cells, thymic epithelium and helper T-cells. Class II MHC molecules are involved in regulating the immune response during tissue graft rejection, stimulation of antibody production, graft-versus-host reactions and in the recognition of "self" (or autologous) antigens, among other phenomena.

"MHC antigens" are defined herein as Class I and/or Class II MHC antigens. MHC antigens of the present invention include both Class I and Class II, either alone or in combination.

"Allogeneic tissue extracts" are defined as splenocyte, splenic tissue or cultured lymphocyte extracts obtained from an allogeneic transplant donor and prepared as described below.

"Immune suppressive fragments" means any peptide or polypeptide containing partial amino acid sequences or moieties of analogs of the relevant MHC antigens possessing the ability to induce suppression of the hosts immune response against organ or tissue allogeneic grafts. Such fragments need not possess the alloantigeneic properties of the entire MHC molecule.

"Analog" of immune suppressive fragments refers to compounds that are structurally related to suppressive fragments of MHC antigens thereof which possess the same biologic activity, i.e., the ability to suppresses a mammalian hosts response against a transplanted organ or tissue. The term includes peptides having amino acid sequences which differ from

the amino acid sequence of the relevant MHC antigens of the potential graft recipient by one or more amino acid residues.

Disease suppressive fragments and analogs for use in the present invention can be synthesized using well known solid phase synthesis techniques (Merrifield, R.B. Fed. Proc. Am. Soc. Ex. Biol. 21: 412, 1962 and J. Am. Chem. Soc. 85: 2149, 1963; Mitchel, A.R. et al., J. Am. Chem. Soc. 98: 7357, 1976; Tam, J. et al., J. Am. Chem. Soc. 105: 6442, 1983). Analogs can be constructed by identifying an equivalent amino acid sequence and using the peptide synthesis techniques disclosed above.

Analogues can be provided using the known amino acid sequence of MHC antigens as disclosed in Immunogenetics 29:231-234, 1989.

Disease-suppressive analogs and fragments can also be obtained using recombinant DNA techniques that are well-known in the art.

Disease suppressive fragments of MHC antigens and analogs thereof can be identified using routine experimentation using suitable in vivo systems such as those of Examples 1-4 below.

T-lymphocytes can be obtained from a potential allograft donor using methods well known in the art and cultured as described in Transplantation 41:549, 1986 and Transplantation 48:639, 1989 and administered to a mammal about to undergo or having undergone (as described below) an organ or tissue allograft.

Extracts (or lysates) of splenic tissue or cultured lymphocytes can be prepared using techniques well known in the art such as those described in Example 1 below.

In accordance with the present invention, conventional
5 tissue typing, well-known in the art and routinely conducted on all transplant donors and recipients, is performed on a potential transplant donor to determine the MHC phenotype of the donor tissue or organ. Synthetic MHC antigens, disease suppressive fragments or their analogs can then be synthesized
10 using the techniques described above. These antigens and/or fragments may be administered to mammals, especially humans, who are to receive a transplant; or to patients that have already received transplanted "non-self" tissue. The methods and compositions of the present invention may be used to treat
15 mammals that have previously received "non-self" organ or tissue transplants and are beginning to display the initial symptoms of allograft rejection (such as fever, tenderness of the transplanted organ or loss of function thereof). The method and compositions of the invention are useful to preserve
20 the organ or tissue and damp down or shut off that portion of the immune response of the recipient that is directed against the transplanted tissue or organ. To be effective the compositions and methods of the present invention must be administered before total rejection occurs.

25 Pursuant to the present invention, MHC antigens or transplantation rejection suppressive fragments or their analogs are ingested by a mammal that is to receive, or has already received a "non-self" organ or tissue transplant via

the oral or enteral route, in an amount of between about 0.1 mg per kg body weight and about 10 mg per kg of body weight per day. The pharmaceutical compositions of the invention may be administered as a single dose or in multiple dose form via the oral or enteral route. Preferably, the is administered in an amount between about 1 mg and about 5 mg per kg body weight of said mammal per day. The exact amount to be administered will vary depending on the severity and stage of a patient's disease and the physical condition of the patient.

When administering splenic cells, cultured lymphocytes or extracts thereof, between about 10^6 and about 10^9 cell equivalents per kg body weight per day may be administered in single or divided doses.

The timing of such treatments shall be such that, if possible, the pharmaceutical formulations or dosage forms of the present invention are administered between about 7 and about 14 days before the transplantation is performed. The treatment is preferably continued for at least about 6 months after the transplanted organ or tissue has been introduced into the host (recipient) organism and may be continued indefinitely if necessary or desirable.

In addition, if a transplant recipient (either already receiving the compositions of the invention or not) begins to manifest symptoms of rejection, the pharmaceutical formulations of the present invention may be administered in increased amounts and/or frequency.

The present invention also is directed to oral dosage forms and pharmaceutical formulations for administration to mammals in order to prolong the survival of or suppress the rejection of a transplanted organ or tissue. It will be understood that any statistically significant prolongation in graft survival pursuant to the treatment of the present invention is within the scope of the invention.

The oral pharmaceutical formulations of the present invention may also contain inert constituents including pharmaceutically acceptable carriers, diluents, fillers, solubilizing or emulsifying agents and salts of the type that are well-known in the art. For example, tablets and caplets may be formulated in accordance with conventional procedures employing solid carriers, such as starch and bentonite, that are well-known in the art. Examples of solid carriers include bentonite, silica, dextrose and other commonly used carriers. Further non-limiting examples of carriers and diluents which may be used in the formulations of the present invention include saline and any physiologically buffered saline solution such as phosphate buffered saline, pH 7-8 and water.

Capsules employed in the present invention may be made from any pharmaceutically acceptable material such as gelatin or cellulose derivatives. The active biological materials of the invention may be administered in the form of sustained release oral delivery systems and/or enteric coated oral dosage forms such as those described in U.S. Patent No. 4,704,292 issued November 3, 1987, U.S. Patent No. 4,309,404 issued

January 5, 1982 and U.S. Patent No. 4,309,406 issued January 5, 1982.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual dose of each dosage form need not in itself constitute an effective amount for suppressing graft rejection since the necessary effective amount can be reached by administration of a plurality of dosage units.

The preferred route of administration of the dosage forms of the present invention is orally or enterally. Preferred oral or enteral pharmaceutical formulations or dosage forms may comprise for example, between about 70 mg and about 500 mg of MHC antigens, disease suppressive fragments or analogs thereof or between about 10^7 - 10^{10} cell equivalents when using allogenic cells or extracts thereof.

In an alternative embodiment of the present invention the pharmaceutical formulations of the present invention are administered to mammals in aerosol form. It is anticipated that smaller quantities of the allogeneic tissue extracts or MHC antigens, disease suppressive fragments or their analogs will be required to achieve suppression of graft rejection when using the aerosol form of administration. This has been found to be the case in treating experimental allergic encephalomyelitis (EAE) with myelin basic protein (MBP), and also in treating adjuvant arthritis with collagen as disclosed in the co-pending U.S. Patent Application of Weiner et al. Ser. No. 454,486 filed December 20, 1989. The quantity of MHC antigens, disease suppressive fragments or the analogs of such

materials which may be administered in an aerosol dosage form would be between about 0.01 mg and 10 mg per kg body weight of a mammal per day. The aerosol dosage forms of the present invention may be administered to a patient via the aerosol route in a single dosage form or multiple dosage forms. The exact amount to be administered will vary depending on the state and severity of a patient's disease, the activity of the patient's immune system and the physical condition of the patient.

When administering splenic cells, cultured lymphocytes or extracts thereof, between about 10^5 and about 10^9 cell equivalents per kg body weight per day may be administered in single or divided doses in an aerosol form.

The aerosol pharmaceutical formulations of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are well-known in the art. Specific non-limiting examples of the carriers and/or diluents that are useful in the aerosol pharmaceutical formulations of the present invention include water, normal saline and physiologically-acceptable buffered saline solutions such as phosphate buffered saline solutions, pH 7.0-8.0.

Examples of useful solubilizing and emulsifying agents are physiologically balanced salt solutions, phosphate buffered saline and isotonic saline. The salts that may be employed in preparing the aerosol dosage forms of the invention include the pharmaceutically acceptable salts of sodium and potassium.

The route of administration of allogeneic spleen cells, cultured lymphocytes extracts thereof or MHC antigen or disease

suppressive fragments or their analogs according to this alternate embodiment of the present invention is in an aerosol or inhaled form. The aerosol compositions of the present invention can be administered as a dry powder or in an aqueous solution. Preferred aerosol pharmaceutical formulations may comprise, for example, a physiologically-acceptable buffered saline solution containing between about 7 mg and about 700 mg of the compositions of the present invention, disease suppressive fragments or analogs thereof.

10 Dry aerosol in the form of finely divided solid particles of tissue extracts from spleen cells, MHC antigens disease suppressive fragments or analogs thereof that are not dissolved or suspended in a liquid are also useful in the practice of the present invention. The compositions of the present invention may be in the form of dusting powders and 15 comprise finely divided particles having an average particle size of between about 1 and 5 microns, preferably between 2 and 3 microns. Finely divided particles may be prepared by pulverization and screen filtration using conventional techniques that are well known to those skilled in the art. The particles 20 may be administered by inhaling a predetermined quantity of the finely divided material, which can be in the form of a dry atomized powder.

 The pharmaceutical formulations of the present invention may be administered via the aerosol route by means of a 25 nebulizer, as an example those described in U.S. Patent Nos. 4,624,251 issued November 25, 1986; 3,703,173 issued November 21, 1972; 3,561,444 issued February 9, 1971 and 4,635,627

issued January 13, 1971. The aerosol material is inhaled by the subject to be treated.

Other systems of aerosol delivery, including for example the pressurized metered dose inhaler (MDI) and the dry powder inhaler as disclosed in Newman, S.P. in Aerosols and the Lung, Clarke, S.W. and Davia, D. eds. pp. 197-224, Butterworths, London, England, 1984 can be used in conjunction with the method of the present invention.

Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, MA), Schering Corp. (Kenilworth, NJ) and American Pharmoseal Co., (Valencia, CA).

In accordance with the present invention, experiments were performed in which the effects of oral administration of allogeneic splenocytes to Lewis rats were studied, with particular attention being given to the effects on the immune response of the transplant recipient. To this end, the in vitro mixed lymphocyte response (MLR), the delayed type hypersensitivity (DTH) reaction, and the in vivo accelerated cardiac allograft rejection techniques were utilized. In each case, the oral administration (to the recipient of a "non-self" tissue transplant) of splenocyte cells from a donor animal resulted in suppression of these T-cell mediated immune reactions. As T-cells have been implicated as the major mediators of allograft rejection, the results of these tests establish the practical efficacy of the methods and pharmaceutical formulations of the present invention.

The present invention is illustrated in specific working examples presented below which are intended to illustrate the present invention without limiting the scope thereof.

5

EXAMPLE 1: PREPARATION OF MATERIALS AND TEST SUBJECTS

1. Subject Mammals

The test population was comprised of male rats of the Lewis (LEW), Wistar Furth (WF) and Brown Norway (BN) variety (obtained from Harlan Sprague Dawley Inc., Indianapolis, IN). The rats in the experiments described below were approximately 8-10 weeks old, and were bred under careful observation.

15

2. Preparation of splenocytes for oral administration

Fresh splenic tissue was obtained from syngeneic (same species, same strain) or allogeneic (same species, different strain) animals shortly prior to oral administration. Single cell splenocyte suspensions were prepared by mashing the fresh spleen through a standard stainless steel mesh (2 inches by 2 inches). Red blood cells were specifically lysed with Tris-ammonium chloride buffer according to standard procedures well known in the art, washed twice with Hank's Balanced Salt Solution (HBSS) and resuspended into various concentrations as described below before use.

25

3. Preparation of splenocyte lysate

Splenocytes prepared as in the above method were lysed by repetitive freeze-thawing in the following manner:

- (a) Cells were quick frozen at -70°C for 30 minutes;
- (b) Quick frozen splenocytes were then thawed at 37°C ;
- 5 (c) This freeze-thaw cycle was repeated one more time.

The resulting materials were used for oral administration.

10 EXAMPLE 2: ORAL ADMINISTRATION OF PREPARED SPLENOCYTE SUSPENSION

A one milliliter dose of the cell suspension as prepared in Example 1, was orally introduced to each test rat with a syringe having a ball-tipped feeding needle (Thomas Scientific, Swedesboro, NJ).

15 The following laboratory immunological and pathological procedures were conducted on the lymphatic organs of the test rats.

EXAMPLE 3: MIXED LYMPHOCYTE REACTION

Cervical lymph nodes were taken from the responder (LEW) and the stimulator (WF or BN) rats. The excised nodes were then pressed through stainless steel mesh as above and suspended in Phosphate Buffered Saline.

20

The isolated lymph node cells were then washed twice and resuspended into RPMI 1640 medium, containing 10% fetal calf serum (FCS), 1% penicillin and streptomycin (Microbiological Associates, Walkersville, MA) 2×10^{-5} M 2-mercaptoethanol, and 5 mM HEPES, at a concentration of 6×10^6 cells/ml.

25

Responder cells were seeded into a 96-well flat-bottomed

culture plates (Costar Cambridge, MA) at 50 microliters per well, with or without irradiated stimulator cells (3000 Rads gamma irradiated using a Shepherd irradiator, Model 143-45 and a Cesium-137 source) of the same volume.

5 The treated cells were then cultured at 37°C with 5% CO₂ for four days before they were pulsed for 6 hours with ³H-thymidine (1 microCi/well, obtained from NEN Dupont, Boston, MA). Cell proliferation was monitored by incorporation of ³H-thymidine measured by a Beckman liquid scintillation counter.

10 SUPPRESSOR ASSAY

 Obtained lymph node cells were irradiated (1000 Rads of gamma radiation) and added to a test MLR at concentrations varying from 5 to 20% of total cells per well (experimental wells). Control wells were set up with no modulators while
15 background wells had only responder cells. These cultures were incubated at 37°C and in 5% CO₂ for 96 hours. Proliferation was assayed by pulsing the plates with 1 microCi/well ³H-thymidine for the last 6 hours of culture. The plates were then harvested as described above.

20 DELAYED TYPE HYPERSENSITIVITY DTH REACTIONS

 Rats of each group were immunized subcutaneously in the footpad with 10 million gamma irradiated (3000 RAD) allogeneic splenocytes. Ten days later, they were injected again with the same dosage in the ear lobe. The responses were determined as
25 the changes in the ear thickness before and 48 hours after the challenge.

CELL TYPING

The phenotypes of the extracted lymphocytes were tested by indirect immunofluorescent staining and with a fluorescence-activated cell sorter (FACS). The lymph cells were first incubated for 1 hour with primary monoclonal antibodies against the cell surface markers CD4 or CD8, or mouse immunoglobulin (Organon-Teknica, Westchester, PA) and washed twice with PBS containing 0.02% sodium azide. They were then further incubated with FITC-conjugated goat-anti-mouse IgG (1:40) (Organon Teknica) in the dark for 30 minutes and in the presence of 15% autologous normal rat serum. The cells were thoroughly washed and fixed with 1% formaldehyde before testing.

Additionally, surgical transplant methods of the type described in the following example were performed.

EXAMPLE 4: CARDIAC ALLOGRAFT

LEW rats were subjected to surgical transplant procedures. An accelerated rejection model was used wherein LEW strain rats were pre-sensitized with BN strain full-thickness skin grafts seven days before the cardiac allograft, with and without oral ingestion of splenocyte preparations.

Seven days later, a (LEWxBN)F1 strain test vascularized cardiac allograft was performed on each pre-treated rat. The cardiac grafts were anastomosed to the infra-renal abdominal aorta. Rejection was defined as complete cessation of heart beat as determined by daily palpation of the recipient's flank.

The above-described methods were used to obtain the following results:

5 I. SUPPRESSION OF THE MIXED LYMPHOCYTE REACTION (MLR) BY ORAL ADMINISTRATION OF ALLOGENEIC SPLENOCYTE PREPARATIONS

Splenocytes from WF rats were freshly prepared and were administered orally to LEW rats two, five or ten times over a 1-2 week period.

10 The individual dosages were 50 million cells per oral administration.

Seven days following the last oral administration, lymph nodes were taken from both a control group and those given oral splenocytes for MLR studies using WF or BN
15 stimulators. As shown in Figure 1, LEW rats which had ingested allogeneic splenocytes showed significantly reduced reaction against the lymphocytes from the WF strain. This phenomenon was observed in all three feeding protocols (i.e., 2, 5 or 10 times). However, only the group that received ten feedings
20 showed suppression against the BN strain, the third party control.

These results indicate that limited ingestion of allogeneic splenocyte preparations induced antigen specific suppression of the MLR.

25

II. COMPARISON OF SUPPRESSION OF MLR BY ORAL ADMINISTRATION OF ALLOGENEIC VERSUS SYNGENEIC SPLENOCYTE PREPARATIONS

A dose response study was subsequently conducted to
30 determine the effect of feeding syngeneic versus allogeneic

cells. LEW rats were fed twice with 1, 5, 25 or 50 million splenocytes from either LEW or WF strains. The results are set forth in Table I below.

TABLE I

5

The Effect of Feeding Syngeneic
and Allogeneic Splenocytes on MLR

Strains used for feeding	Dosage cells/feeding	MLR delta/CPM	Relative Response (%)
10	----	115015±7707	100
LEW	1 X 10 ⁶	128520±8338	112
	5 X 10 ⁶	54391±10988	47
	25 X 10 ⁶	39088±7294	34
15	50 X 10 ⁶	81329±8013	71
WF	1 X 10 ⁶	71135±13721	62
	5 X 10 ⁶	79011±14119	68
	25 X 10 ⁶	56196±15254	49
	50 X 10 ⁶	73541±11636	64
20			

Feeding at the lowest dosage (1 million) of syngeneic cells did not induce suppression; all other doses, both syngeneic and allogeneic cells, show some suppression to varying degrees.

25

III. EFFECT OF INGESTED LYSATE OF ALLOGENEIC
SPLENOCYTE PREPARATIONS ON MLR

The effect of ingested lysate alone on MLR was next studied to determine whether live splenocytes were required for

the orally induced tolerance. Rats were given two separate oral doses of either live splenocytes or the corresponding lysate prepared by the repetitive freeze and thaw method (described above) and the effect of these treatments were compared. Figure 2 shows that cell lysate alone was sufficient in suppressing the MLR, indicating that a subcellular fragment was involved in suppressing the cell-mediated immunity.

IV. KINETICS OF MLR SUPPRESSION BY ORAL ADMINISTRATION OF ALLOANTIGENS

The kinetics of the orally induced tolerance to alloantigen was studied by giving two oral doses of splenocytes to separate LEW rat groups, 14 days, 7 days, 3 days, and 1 day before the MLR was performed. As shown in Figure 3, the groups which were given oral doses 1 day or 3 days before MLR was performed did not induce suppression. The groups with 7-day and 14-day intervals between the last oral ingestion and MLR showed dramatic reduction of proliferation in MLR, indicating that more than 4 days were required for the induction of oral unresponsiveness to alloantigens.

V. SUPPRESSION OF DTH RESPONSE AGAINST ALLOANTIGENS

In addition to the in vitro MLR, the effect of ingesting allogeneic splenocytes on the delayed type hypersensitivity (DTH) response, in vivo, in LEW rats was examined. LEW rats were orally administered 10 feedings of 50 million splenocytes from either syngeneic or allogeneic (WF) animals. After the last oral ingestion, the test for DTH was initiated with the animals being immunized subcutaneously in their foot pads. The

same animals were injected again 10 days later in the ear lobes. The DTH was measured as the changes in the ear thickness before and 48 hours after the challenge. The results are shown in Figure 4.

5 Approximately 50% decrease in DTH response to WF was observed in rats fed with cells of the same strain, but not in those fed with syngeneic LEW splenocytes. The DTH response against BN was not affected by the pre-treatment, indicating that the DTH suppression was antigen specific.

10 VI. ACTIVE SUPPRESSION IS INVOLVED IN
 MEDIATING DECREASED PROLIFERATION IN THE MLR

 In order to study the mechanism of inhibition of MLR proliferation in the fed animals, a suppressor cell assay was performed to determine if CD8+ suppressor cells were involved
15 in mediating the observed effects. Lymphocytes from either control or pre-fed animals were irradiated with 1000 RADS of gamma radiation before being added to a primary MLR, serving as modulators.

 Lewis rats (3/group) were pre-treated 10 times orally
20 with varying dosages (as indicated in the Table) of WF splenocytes. One week later, their cervical lymph nodes were taken and the cells served as modulator after being irradiated 1000 Rad of gamma radiation. The primary LEW anti-WF and LEW anti-BN MLR and Con-A stimulation cultures were set up as
25 described above. Modulator cells were added to the primary cultures at a 1/5 ratio. The results are set forth in Table II below.

TABLE II

**SUPPRESSION OF PRIMARY MLR BY LYMPHOCYTES FROM
ALLOGENEIC SPLENOCYTES FED RATS**

Source of modulator	anti-WF		anti-BN		Con A	
	CPM (X10 ⁻³)	% Supp.	CPM (X10 ⁻³)	% Supp.	CPM (X10 ⁻³)	% Supp.
--	112±21		405±78		280±4.7	
control	104±11	7	464±15	0	276±6.6	1.4
fed 10X10 ⁶	106±17	5.3	334±120	17	277±34	1
fed 25X10 ⁶	84±15	25	443±17	0	305±3.9	0
fed 50X10 ⁶	1±0.9	99	84±7.1	79	199±12	29

The results in Table II show that adding 20% of modulators from pre-fed animals, but not from the control animals, suppressed the primary LEW-anti-WF MLR. This suggests that suppressor cells were induced after feeding and these in turn mediated suppression of the MLR.

**VII. PHENOTYPE OF LYMPH NODE CELLS FROM
FROM ANIMALS INGESTING SPLENOCYTES**

Cervical lymphocytes from either control or fed animals were cultured with irradiated WF stimulators for 5 days, then sorted for CD4+ or CD8+ cells by indirect immunofluorescence staining. The results shown in Figure 5 show that pre-feeding rats with allogeneic splenocytes resulted in an increase in CD8+ (suppressor T-cells) cells and a decrease in CD4+ (helper T-cells) cells when compared to controls.

VIII. ORAL ADMINISTRATION OF SPLENOCYTES PREVENTS
ACCELERATED CARDIAC ALLOGRAFT REJECTION

To demonstrate the prevention of allograft rejection,
5 an accelerated rejection transplantation model, as described
above, was used. LEW rats were pre-sensitized with BN skin
grafts 7 days before challenge with vascularized BN test
cardiac allografts, to study the effects of feeding allogeneic
donor splenocytes on test graft survival.

10 While unsensitized controls rejected their cardiac
allografts on the 6th through the 8th day, all sensitized
control animals hyperacutely rejected their cardiac allografts
within 36 hours. Test animals fed 5-10 feedings of 50 million
splenocytes, 7 days prior to the skin graft, or even on the day
15 of the skin graft, exhibited increased test cardiac allograft
survival, to 7.62 ± 0.5 days.

These results show that feeding allogeneic splenocytes
prevents sensitization and converts accelerated rejection into
an acute form.

20 The specificity of this phenomenon was examined as
described below.

Cardiac recipient LEW rats were either unfed (n=10),
fed LEW (syngeneic) lymphocytes (n=8), fed BN splenocytes (but
received a WF cardiac allograft, n=6) or were fed BN
25 splenocytes (and received a BN cardiac allograft, n=8). All
fed animals received 5-10 feedings of 50×10^6 splenocytes.
The results are shown in Figure 6.

As can be seen in Figure 6, only the rats which were fed allogeneic splenocytes showed cardiac allograft survival beyond day 3. LEW rats fed third party (BN) lymphocytes but receiving a WF graft did not demonstrate enhanced cardiac allograft survival, demonstrating the specificity of this reaction.

In a preliminary attempt to study the mechanism of graft prolongation, the MLR of cervical lymph node cells from control and fed sensitized LEW rats were examined at 48 hours after the cardiac transplant. The results are shown in Figure 7.

There was a suppression of the MLR in the fed animals as compared to the control (Figure 7). These data are consistent with the previous MLR findings in the naive animal model.

WHAT IS CLAIMED IS:

1 1. A method for suppressing the immune response of a
2 recipient mammal to the presence of non-self tissue from a
3 donor mammal comprising:

4 orally or enterally administering to a mammal in
5 need of such treatment an immune suppressive effective amount
6 of an agent specific for suppressing said immune response.

1 2. The method of claim 1 wherein said agent is a
2 member selected from the group consisting of splenocytes from
3 said donor, splenic extracts from said donor, cultured lym-
4 phocytes from said donor, extracts of said cultured lym-
5 phocytes, MHC antigens from said donor, disease suppressive
6 fragments of said MHC antigens and analogs thereof from said
7 donor.

1 3. A pharmaceutical formulation for administration to
2 a recipient mammal about to undergo or having undergone
3 transplant surgery from a donor mammal, comprising:
4 an oral dosage form containing an effective amount
5 for suppressing transplantation rejection of an agent selected
6 from the group consisting of splenic tissue from said donor,
7 extracts of said splenic tissue, cultured lymphocytes from said
8 donor, extracts of said cultered lymphocytes, transplantation
9 MHC antigens from said donor, disease suppressive fragments of
10 said MHC antigens and analogs thereof.

1 4. The pharmaceutical formulation of claim 3 wherein
2 said oral dosage form is a solid dosage form selected from the
3 group consisting of a tablet, a capsule and a caplet.

1 5. The pharmaceutical formulation of claim 3 wherein
2 the oral dosage form comprises an aqueous suspension solution.

1 6. The pharmaceutical formulation of claim 3 compris-
2 ing a pharmaceutically acceptable carrier or diluent.

1 7. A method for suppressing the immune response of a
2 recipient mammal to the introduction of a donor's foreign
3 tissue comprising:
4 aerosol administering to a mammal in need of such
5 treatment an immune suppressive effective amount of an agent
6 selected from the group consisting of splenocytes from said
7 donor, splenic extracts from said donor, cultured lymphocytes
8 from said donor, extracts of said cultured lymphocytes, MHC
9 antigens from said donor, disease suppressive fragments of MHC
10 antigens and analogs thereof.

1 8. A pharmaceutical formulation for administration to
2 a recipient mammal about to undergo or having undergone
3 transplant surgery from a donor mammal, comprising:
4 an aerosol dosage form containing an effective
5 amount for suppressing transplantation rejection of an agent
6 selected from the group consisting of splenic tissue from said
7 donor, extracts of said splenic tissue, cultured lymphocytes

8 from said donor, extracts of said cultured lymphocytes,
9 transplantation MHC antigens from said donor, disease suppres-
10 sive fragments of said MHC antigens and analogs thereof.

1 9. The pharmaceutical formulation of claim 8 wherein
2 the aerosol dosage form comprises an aqueous suspension
3 solution.

1 10. The pharmaceutical formulation of claim 8 compris-
2 ing a pharmaceutically acceptable carrier or diluent.

1 11. A method for suppressing graft rejection in a
2 mammal comprising administering to a mammal in need of such
3 treatment an effective amount of an agent which specifically
4 suppresses said mammals immune response directed against said
5 graft.

1 12. An oral dosage form for administration to a mammal
2 comprising a composition containing an agent that specifically
3 suppresses said mammal's immune response directed against a
4 transplanted organ or tissue.

1 13. A dosage form for administration to a mammal via
2 the oral cavity comprising a composition containing an agent
3 which specifically suppresses said mammal's immune response
4 directed against a transplanted organ or tissue.

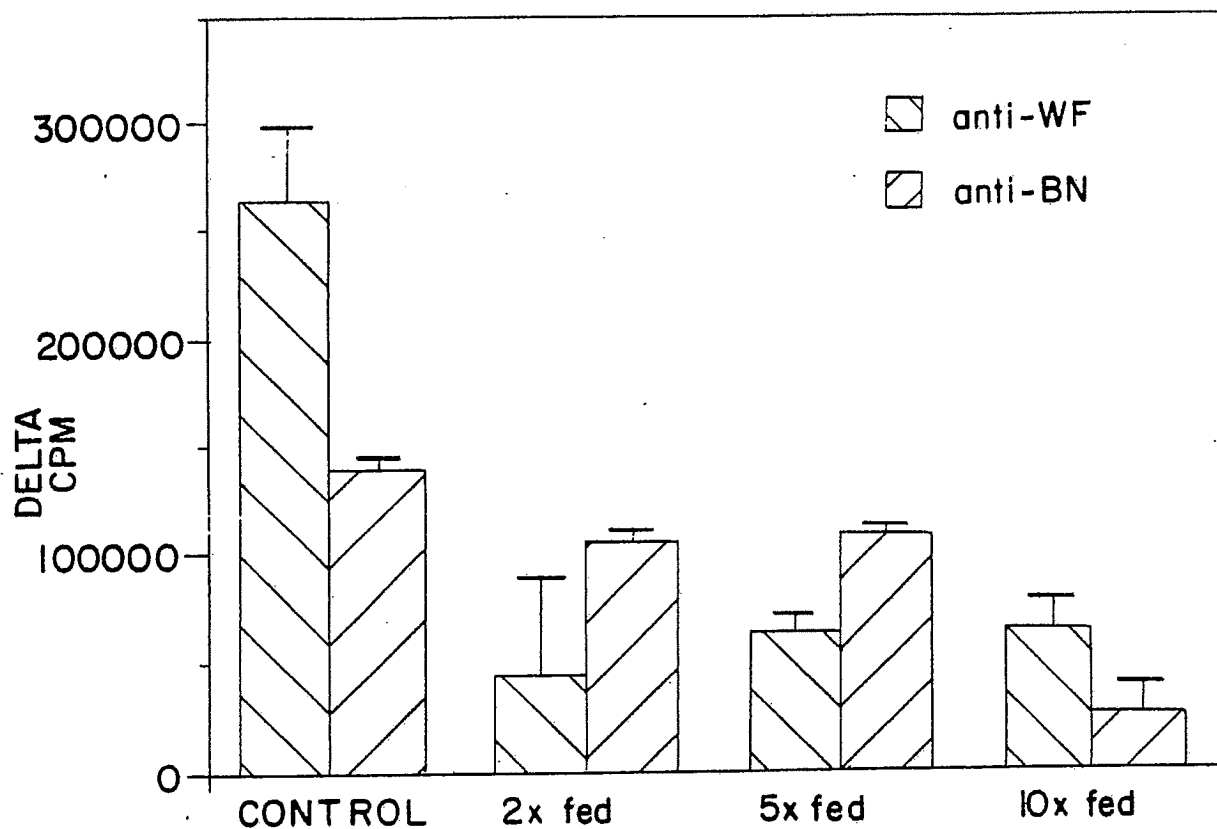


FIG. 1

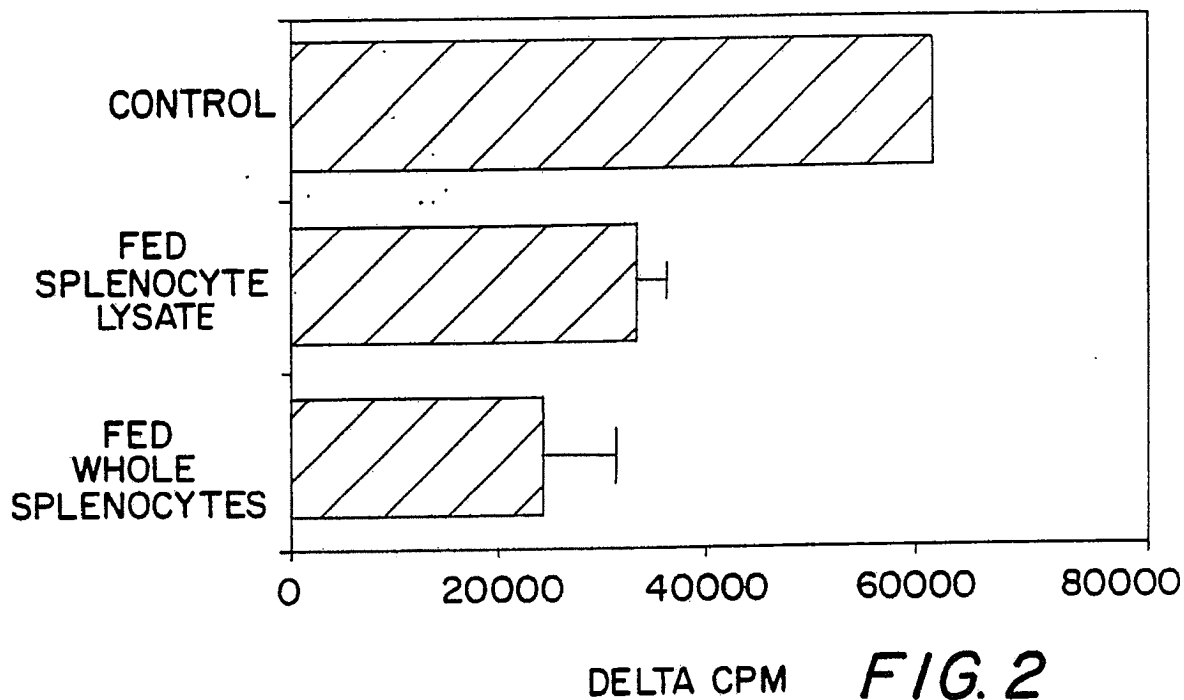


FIG. 2

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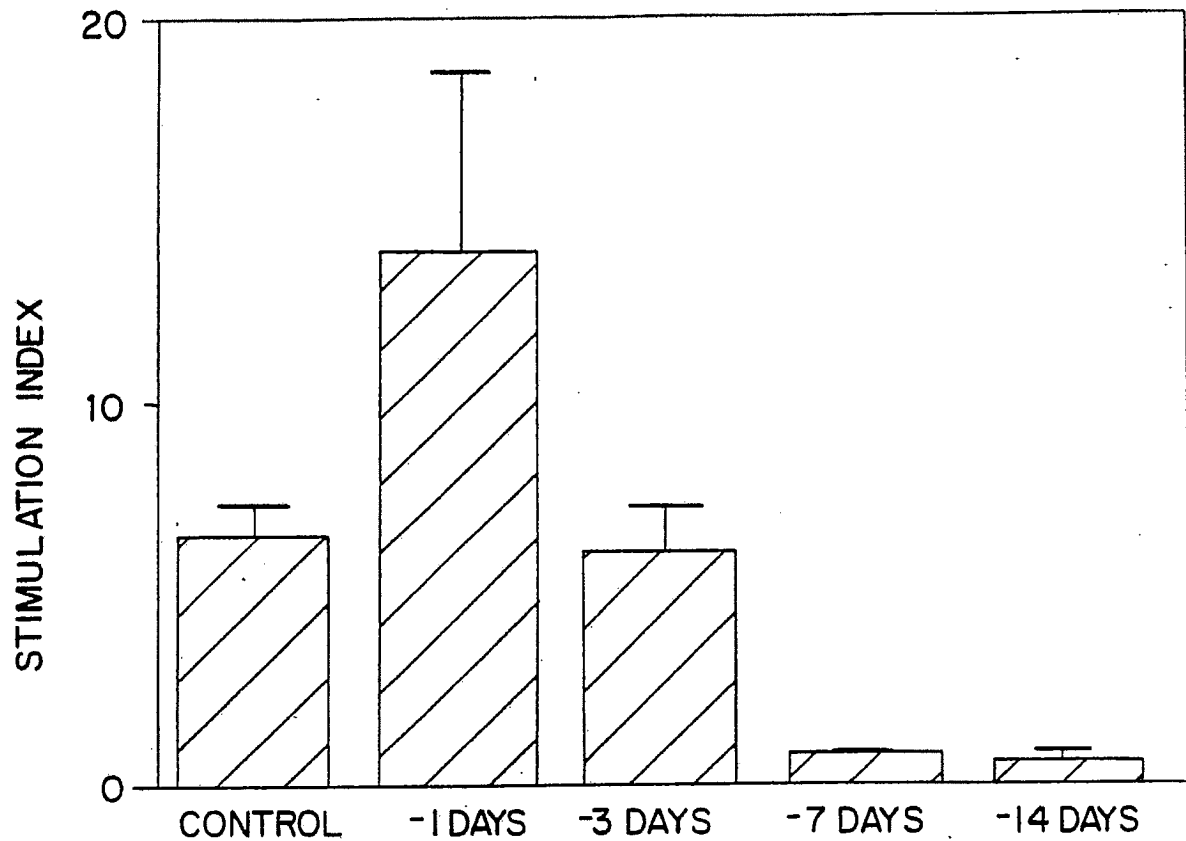


FIG. 3

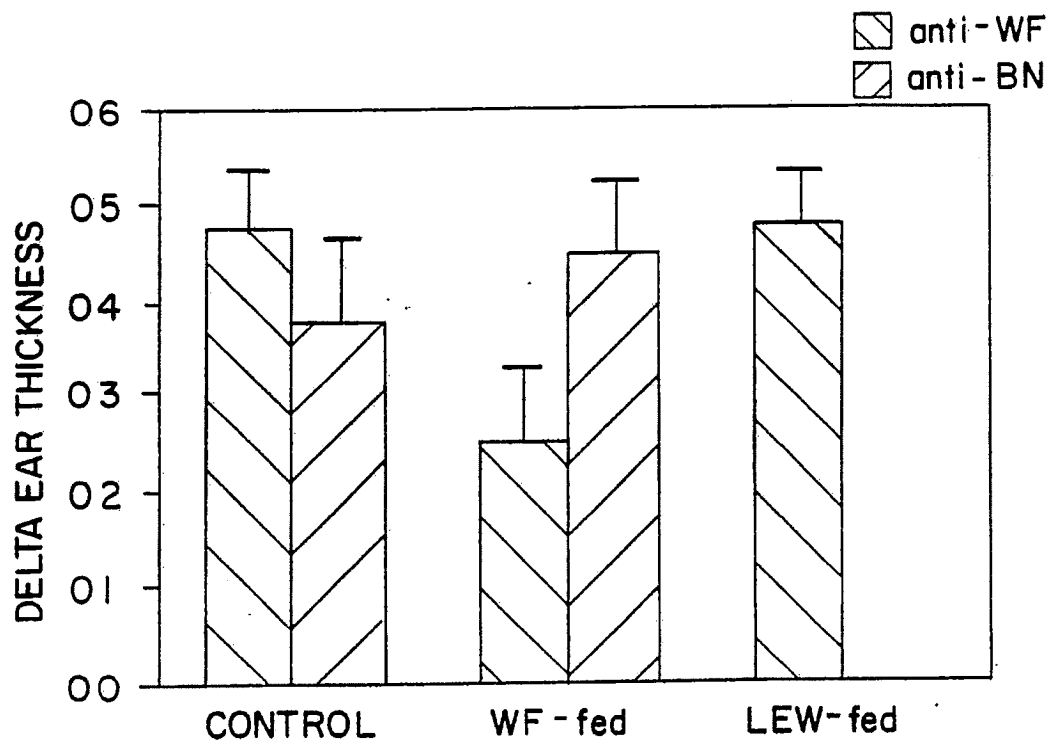
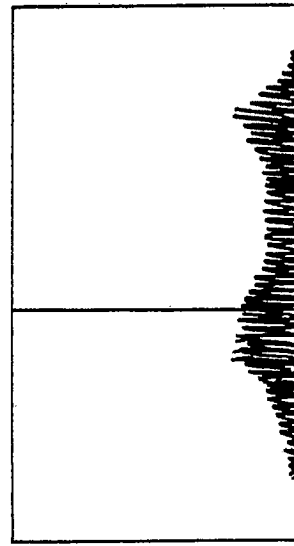
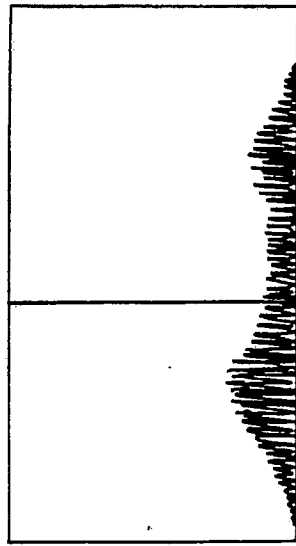
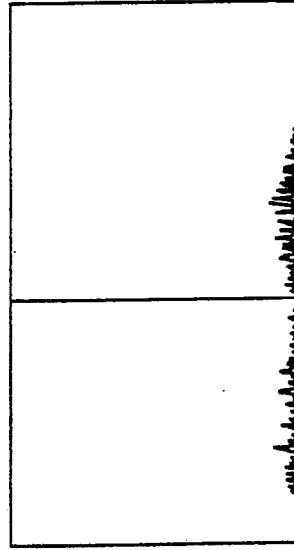
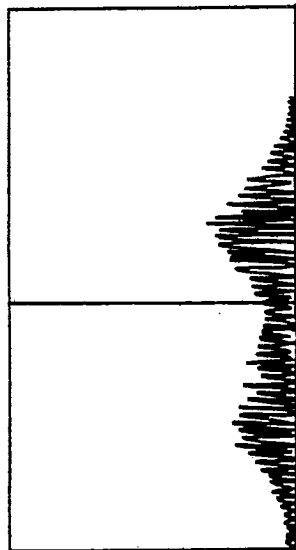


FIG. 4

3/5



CD4+



CD8+

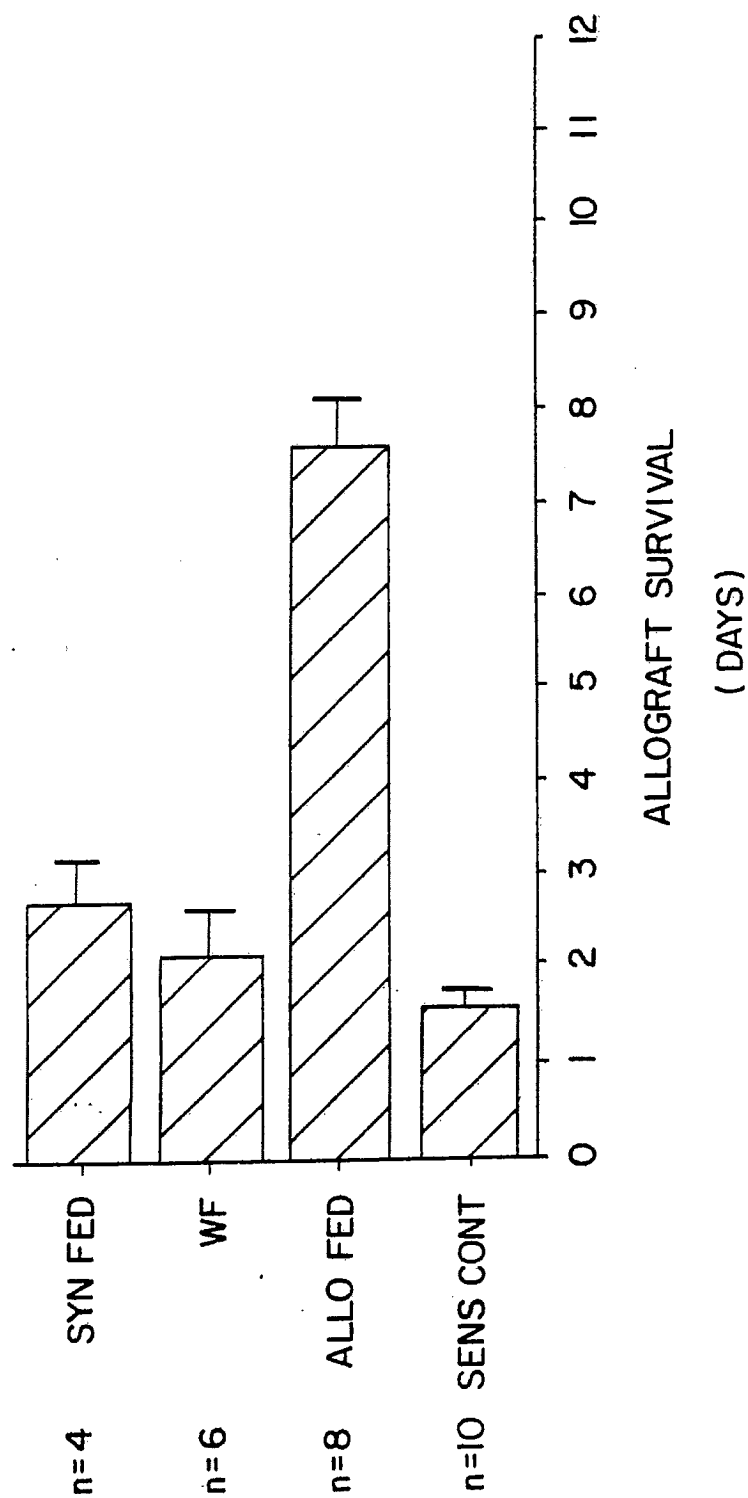
CONTROL

FED

FIG. 5

4/5-

FIG. 6



5/5

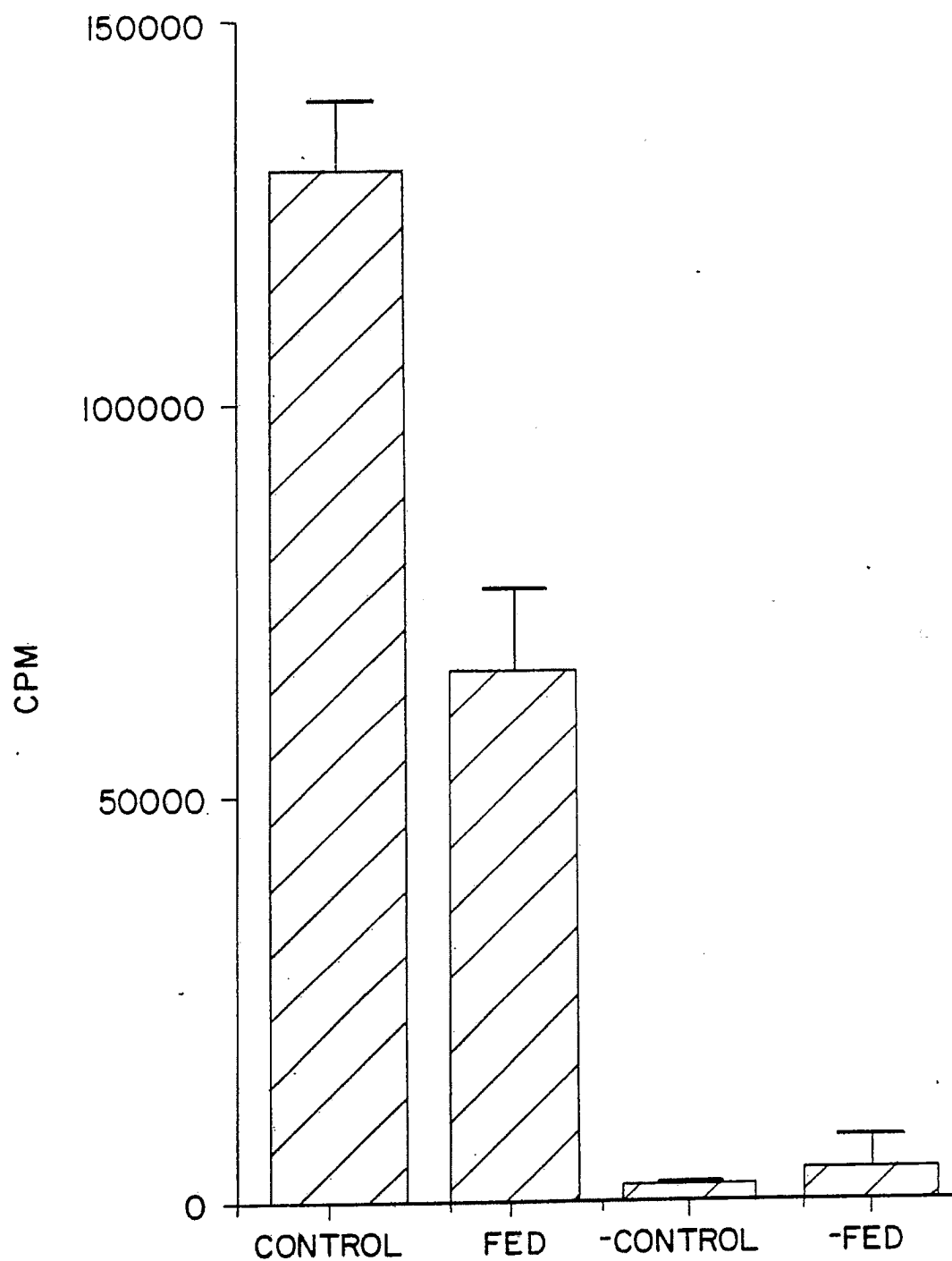


FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08143

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): ~~A61K~~ 39/00, 37/10, 37/00

U.S.CL: 424/88; 514/8, 21

II. FIELDS SEARCHED

Minimum Documentation Searched ?

Classification System

Classification Symbols

U.S.

424/88;

514/8, 21

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

Databases: Dialog (Files 5, 155, 154, 399, 357, 172, 173), USPTO
Automated Patent System (File USPAT, 1971-1991.)

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category * Citation of Document, ** with indication, where appropriate, of the relevant passages ** Relevant to Claim No. 13

Y

Transplantation, Volume 39, No. 1, issued
January 1985, Wood et al., "Suppression of
Renal Allograft Rejection In the Rat By
Class I Antigens On Purified Erythrocytes,"
pages 56-62, see abstract.

1-13

Y

The Journal of Immunology, Volume 121, No.
6, issued December 1978, Richman, et al.,
"Enterically Induced Immunologic Tolerance I.
Induction of Suppressor T Lymphocytes by
Intragastric Administration of Soluble
Proteins," pages 2429-2434, see abstract.

1-13

* Special categories of cited documents: **

"A" document defining the general state of the art which is not
considered to be of particular relevance

"E" earlier document but published on or after the international
filing date

"L" document which may throw doubts on priority claim(s) or
which is cited to establish the publication date of another
citation or other special reason (as specified)

"C" document referring to an oral disclosure, use, exhibition or
other means

"P" document published prior to the international filing date but
later than the priority date claimed

"T" later document published after the international filing date
or priority date and not in conflict with the application but
cited to understand the principle or theory underlying the
invention

"X" document of particular relevance; the claimed invention
cannot be considered novel or cannot be considered to
involve an inventive step

"Y" document of particular relevance; the claimed invention
cannot be considered to involve an inventive step when the
document is combined with one or more other such docu-
ments, such combination being obvious to a person skilled
in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

02 January 1992

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

10 Feb 1992

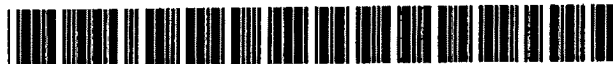
Signature of Authorized Officer

Lynette F. Smith

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The Journal of Immunology, Volume 140, No. 2, issued 15 January 1988, Higgins et al., "Suppression of Experimental Autoimmune Encephalomyelitis by Oral Administration of Myelin Basic Protein and Its Fragments", pages 440-445, see entire article.	1-13
Y	Clarke and Davis, "Aerosols and The Lung" published 1984, see pages 197-224, especially pages 199 and 218.	1-13

EXHIBIT 22



US005788968A

United States Patent [19]

Weiner et al.

[11] **Patent Number:** **5,788,968**[45] **Date of Patent:** ***Aug. 4, 1998**[54] **METHODS AND COMPOSITIONS FOR SUPPRESSING ALLOGRAFT REJECTION IN MAMMALS**[75] Inventors: **Howard L. Weiner**, Brookline; **David Allen Hafler**, West Newton; **Charles B. Carpenter**, Weston; **Mohamed Sayegh**, Brookline; **Zhengyi Zhang**, Malden, all of Mass.[73] Assignee: **AutoImmune, Inc.**, Lexington, Mass.

[*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 5,593,698.

[21] Appl. No.: **461,661**[22] Filed: **Jun. 5, 1995****Related U.S. Application Data**

[63] Continuation of Ser. No. 159,044, Nov. 29, 1993, Pat. No. 5,681,556, which is a continuation of Ser. No. 989,884, Dec. 10, 1992, abandoned, which is a continuation of Ser. No. 607,826, Oct. 31, 1990, abandoned.

[51] Int. Cl.⁶ **A61K 39/00; A61K 39/38; C07K 1/00; C07K 14/00**[52] U.S. Cl. **424/184.1; 424/185.1; 530/350**[58] Field of Search **424/184.1; 530/350**[56] **References Cited****U.S. PATENT DOCUMENTS**

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US91/08143 5/1992 WIPO .

US93/03708 10/1993 WIPO .

OTHER PUBLICATIONSHancock, W.W. et al., *Transplantation* 55:1112-8, 1993.
Sayegh, M.H. et al., *American Society of Histocompatibility and Immunogenetics*, 18th Annual Meeting, Abstract, Oct. 1992.Sayegh, M.H. et al., *American Society of Nephrology*, 25th Annual Meeting, Abstract, Nov. 1992.Sayegh, M.H. et al., *Proc. Nat'l. Acad. Sci. (U.S.A.)* 89: 7762-9, 1992.Sayegh, M.H. et al., *Transplantation* 53:162-6, 1992.Wood et al., *Transplantation* 39(1):56-61, 1985.Tilney, et al., *J. Immunology* 121(14):1480-1482, 1978.Milton et al., *J. Exp. Med.* 161:98-112, 1985.*Remington's Pharmaceutical Sciences*, 1633-1634, 1658-59, 1664-65, 1694-95, 1948.Mason D.W. et al., *Ann. Rev. Immunol.* 4:119-145, 1986.Mowat et al., *Immunol. Today* 8(3):93-98, 1987.Chao et al., *Immunogenetics* 29:231-234, 1989.Krensky et al., *New Engl. J. Med.* 32:510-517, 1990.Wood, et al. 1985, *Transplantation* 39(1): 56-61.Richman, et al. 1978, *Journal of Immunology* 140(2): 2429-2434.Higgins, et al. 1988, *Journal of Immunology* 140(2): 440-445.Newman, 1984, *Therapeutic Aerosols*, Clarke and Davis eds. *Aerosols and The Lung* 197-224.*Primary Examiner*—Lynette F. Smith
Attorney, Agent, or Firm—Darby & Darby

[57]

ABSTRACT

Disclosed herein are methods for suppressing allograft rejection in mammals comprising administering to a mammal about to undergo or having undergone allograft surgery an agent selected from the group consisting of splenic tissue from an allograft donor, splenic extracts, cultured lymphocytes from an allograft donor, extracts of said cultured lymphocytes, MHC antigens, transplantation rejection suppressive fragments and analogs of MHC antigens in an oral or aerosol form. Also disclosed herein are pharmaceutical formulations and dosage forms for use in said methods.

6 Claims, 6 Drawing Sheets

FIG. 1

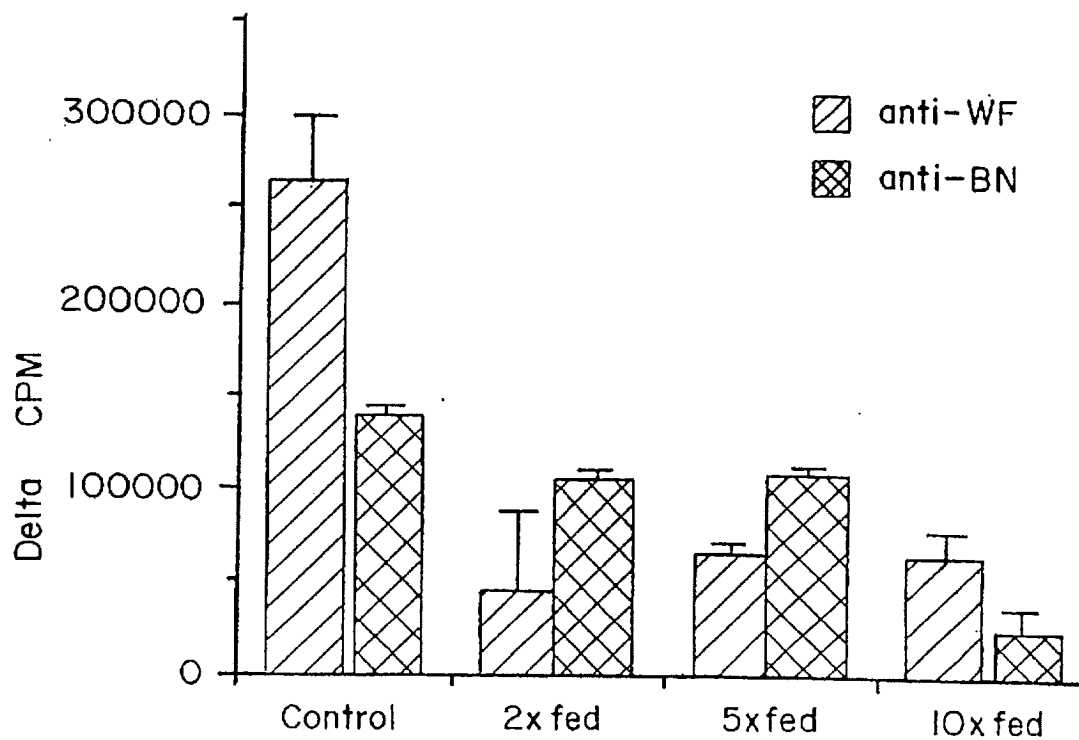


FIG. 2

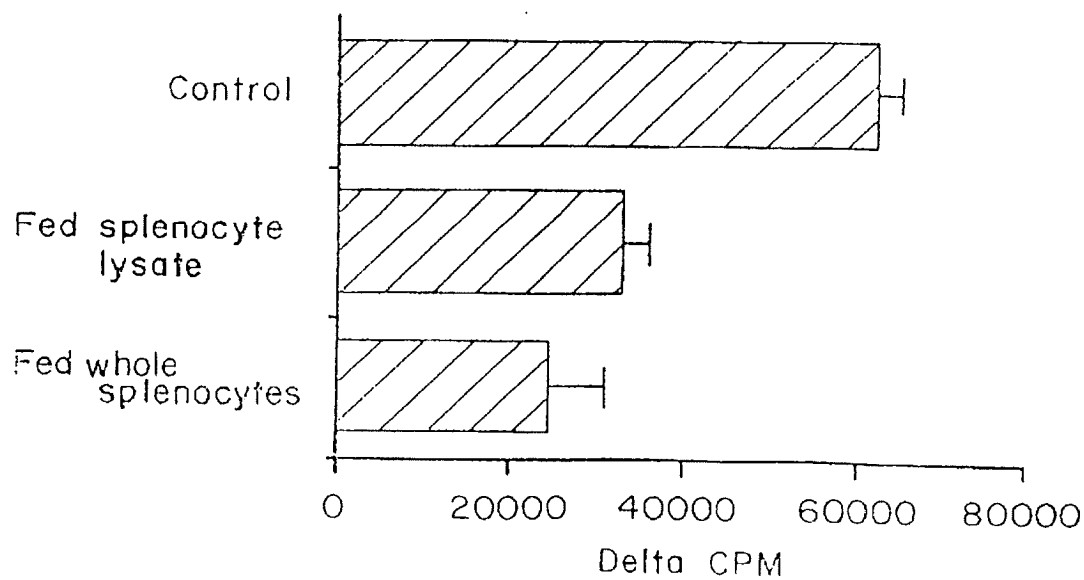


FIG. 3

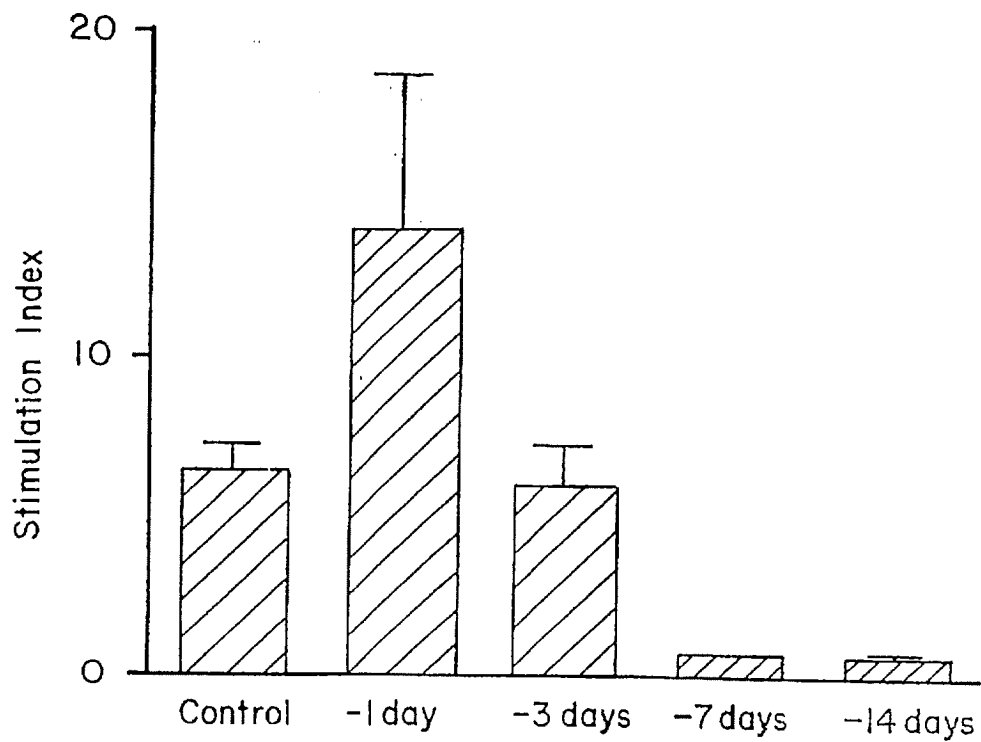


FIG. 4

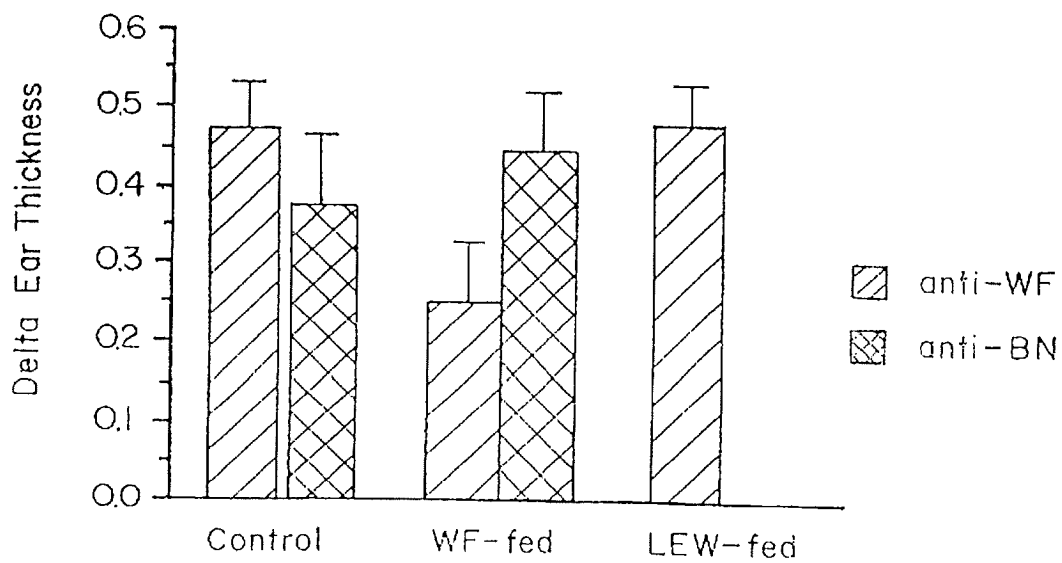


FIG. 5A

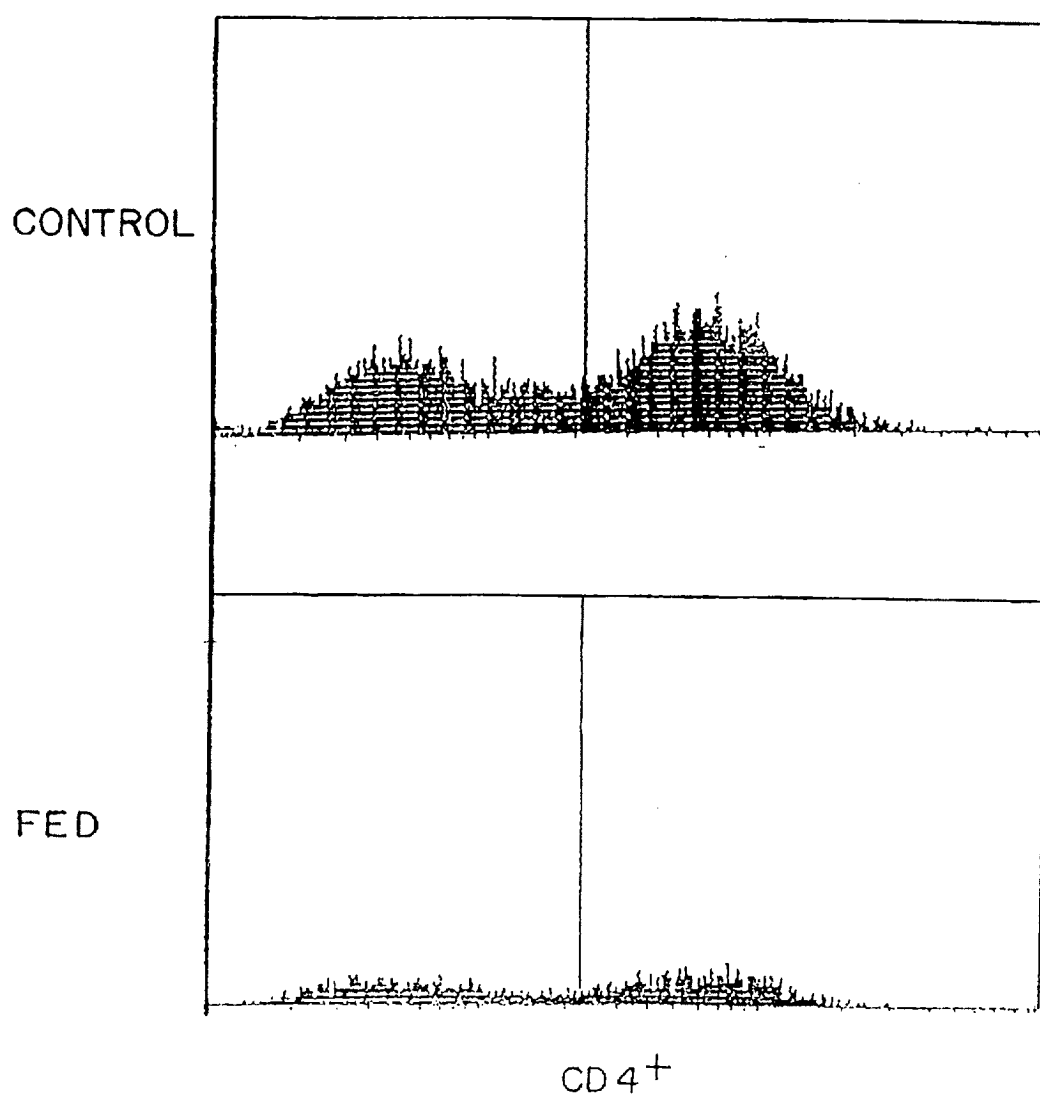


FIG. 5B

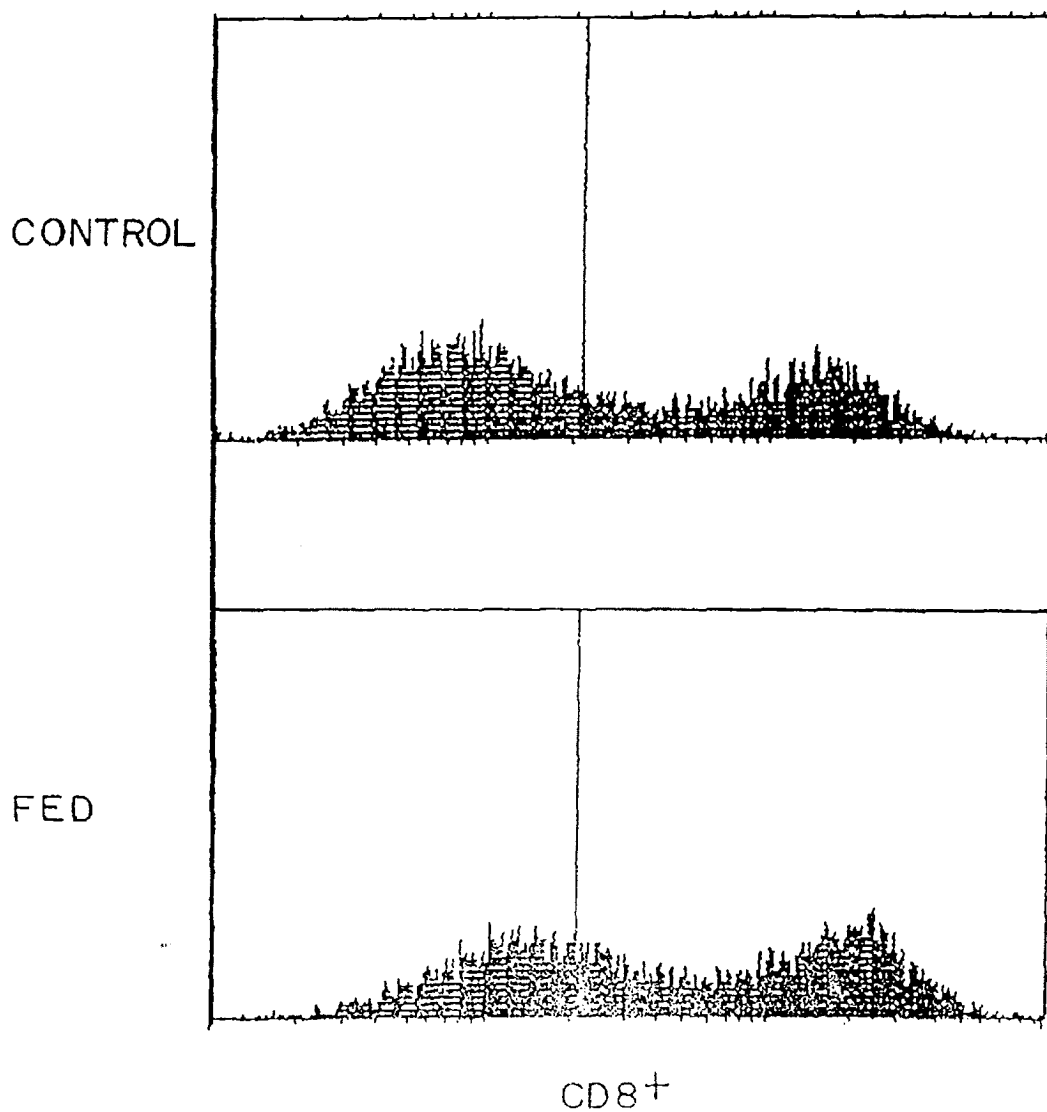
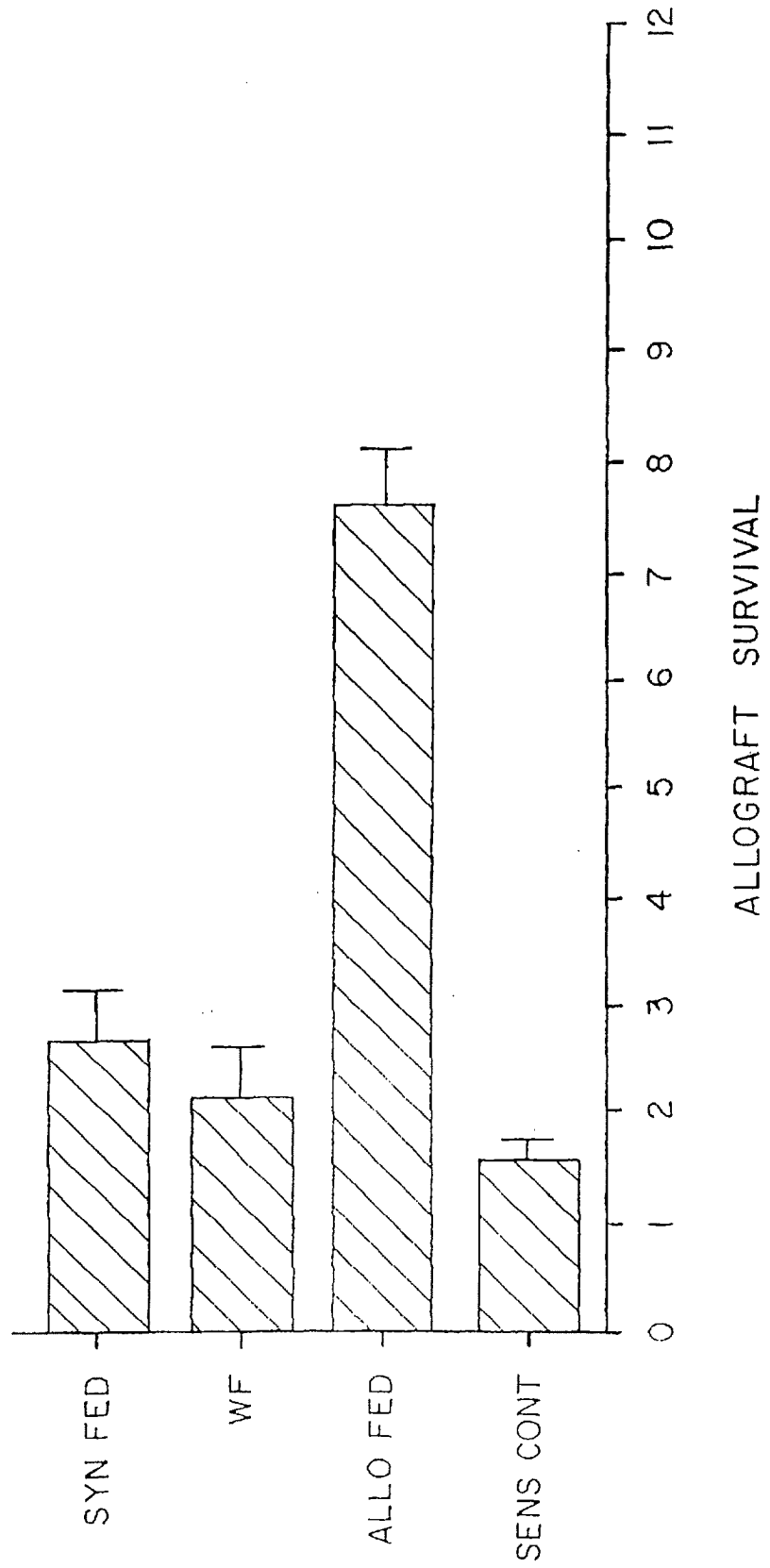
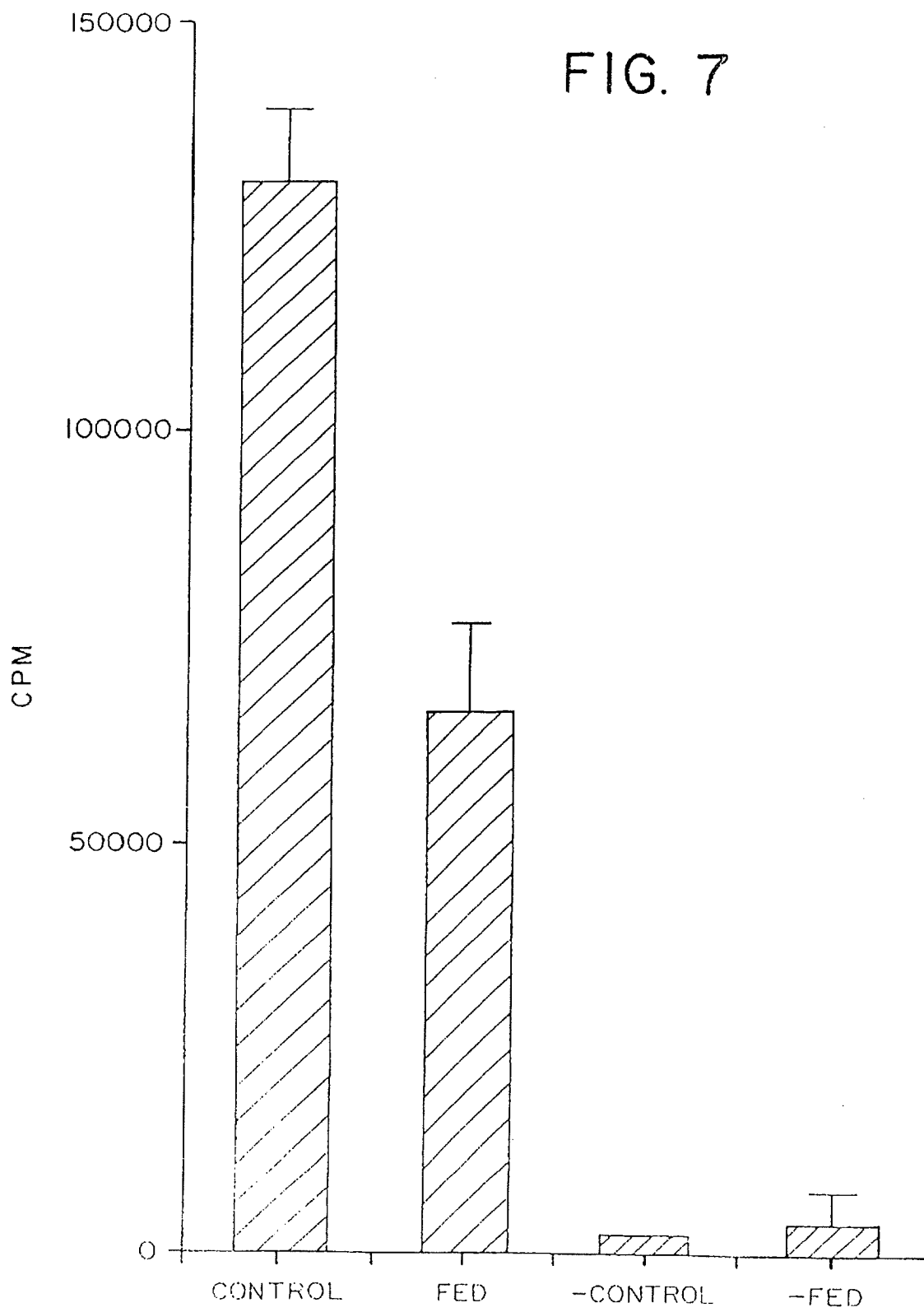


FIG. 6





METHODS AND COMPOSITIONS FOR SUPPRESSING ALLOGRAFT REJECTION IN MAMMALS

This is a continuation of application Ser. No. 08/159,044 filed Nov. 29, 1993 (now U.S. Pat. No. 5,681,556), which is a continuation of application Ser. No. 07/989,884 filed Dec. 10, 1992 now abandoned, in turn a continuation of Ser. No. 07/607,826 filed Oct. 31, 1990 now abandoned.

FIELD OF THE INVENTION

This invention relates to methods and compositions for suppressing the immune response in animals. More particularly, but not by way of limitation, the present invention is directed to pharmaceutical formulations and methods for suppressing and controlling the immune response of mammals against the introduction of foreign tissue. The invention also includes methods for prolonging the survival of transplanted organs and tissues.

BACKGROUND OF THE INVENTION

The success of surgical transplantation of organs and tissue is largely dependent on the ability of the clinician to modulate the immune response of the transplant recipient. Specifically the immunological response directed against the transplanted foreign tissue must be controlled if the tissue is to survive and function. Currently, skin, kidney, liver, pancreas and heart are the major organs or tissues with which allogeneic transplantations are performed. It has long been known that the normally functioning immune system of the transplant recipient recognizes the transplanted organ as "non-self" tissue and thereafter mounts an immune response to the presence of the transplanted organ. Left unchecked, the immune response will generate a plurality of cells and proteins that will ultimately result in the loss of biological functioning or the death of the transplanted organ.

Tissue and organ transplant recipients are customarily treated with one or more cytotoxic agents in an effort to suppress the transplant recipient's immune response against the transplanted organ or tissue. For example, cyclosporin (cyclosporin A), a cyclic polypeptide consisting of 11 amino acid residues and produced by the fungus species *Tolypocladium inflatum* Gams, is currently the drug of choice for administration to the recipients of allogeneic kidney, liver, pancreas and heart (i.e., wherein donor and recipient are of the same species of mammals) transplants. However, administration of cyclosporine is not without drawbacks as the drug can cause kidney and liver toxicity as well as hypertension. Moreover, use of cyclosporin can lead to malignancies (such as lymphoma) and lead to opportunistic infection due to the "global" nature of the immunosuppression it induces in patients receiving long term treatment with the drug, i.e., the hosts normal protective immune response to pathogenic microorganisms is downregulated thereby increasing the risk of infections caused by these agents.

Preliminary results have shown FK-506 (which has a similar mode of action as cyclosporine) to be as potent as cyclosporin in its immunosuppressive qualities and to have fewer toxic side effects than cyclosporin. However, because studies on FK-506 are only in the early stages, it is not available to the general population. Hence, the use of this agent is limited.

Other drugs and/or therapies which are currently administered (either in conjunction with cyclosporin or alone) to suppress the rejection of allogeneic grafts or allografts are also non-specific immunosuppressive drugs or therapies.

Steroids, such as prednisone and methylprednisolone, and Azathioprine (an analog of 6-mercaptopurine) are among the non-specific immunosuppressive drugs used to prolong allograft survival in transplantation recipients.

OKT3 monoclonal antibodies, directed against the CD3 antigen present on T-cells, have also been employed as non-specific immunosuppressive therapeutic agents in allograft recipients. However, OKT3 monoclonal antibodies are of murine origin and the patients to whom such monoclonal antibodies are given mount an immune response against these foreign proteins. Thus the usefulness of such materials is limited.

Another drawback to the above-mentioned drugs and antibodies is that they must be administered indefinitely to suppress allogeneic graft rejection, and tolerance to the foreign tissue does not develop.

Total lymphoid irradiation (TLI) is yet another form of non-specific immunosuppressive therapy that has been used clinically and experimentally to prolong allograft survival. The radiation exposure and treatment schedule for TLI were developed for the treatment of Hodgkin's disease and were subsequently found to be immunosuppressive. Although, TLI induces production of the "global" immunosuppression mentioned above and has the same limitations of other global immunosuppressive therapies, it is the only form of immunosuppression currently in use which appears to induce a specific tolerance to allogeneic tissue. However, TLI is cumbersome to administer and is in an early stage of development, and thus its usefulness is limited.

The oral and aerosol administration of antigens has also been recognized as an effective way to suppress the immune response in mammals to these antigens. The advantages of administering antigens via the oral route include: the simplicity of the techniques involved; the convenience of such techniques since many of the methods can be developed in-situ at the research or treatment facility; the safe, non-toxic effects of the ingestion route; and the specificity that can be provided with the antigens.

Recent studies on several autoimmune disease models have demonstrated that the oral administration of antigens can suppress at least the portion of the immune response that is directed against autoantigens and also protect the treated animals from the induction of specific autoimmune diseases. For example, various animal models are available for the study of Type 1 diabetes as an autoimmune disorder. These include the BB rat (Nakbookda, A. F., et al., *Diabetologia* 14: 199-207, 1978) and the NOD (non-obese diabetic) mouse in which diabetes develops spontaneously (Prochazka et al. *Science* 237:286, 1987). Islet-cell specific, CD4- and CD8-positive T-lymphocytes have been implicated as the causative agents responsible for damage to islet beta cells, as demonstrated by transfer of lymphocytes from affected adults to newborn animals (*J. Exp. Med.* 16-823, 1987).

Experimental allergic encephalomyelitis (EAE) is an induced T-cell mediated autoimmune disease directed against myelin basic protein (MBP) that is widely used as an animal model for the human disease Multiple Sclerosis (MS). EAE can be induced in small mammals by intravenous administration of MBP and a strong adjuvant, such as Freund's complete adjuvant. This treatment induces an acute, monophasic autoimmune disease with the characteristics of MS.

Weiner et al., U.S. patent application Ser. No. 07/595,468 entitled Method Of Treating Or Preventing Type 1 Diabetes By Oral Administration Of Insulin, filed Oct. 10, 1990 now

abandoned, discloses oral and aerosol compositions and pharmaceutical formulations containing insulin which are useful for treating mammals suffering from or at risk for autoimmune diseases having the characteristics of Type 1 diabetes.

Weiner et al., U.S. patent application Ser. No. 460,852 filed Feb. 21, 1990 now abandoned, (the national stage of PCT Application No. PCT/US88/02139, filed Jun. 24, 1988), which is a continuation-in-part application of U.S. patent application Ser. No. 065,734 filed Jun. 24, 1987 now abandoned, generally discloses the treatment of autoimmune diseases by oral administration of autoantigens.

Weiner et al., U.S. patent application Ser. No. 454,806 filed Dec. 20, 1989 now abandoned, discloses the aerosol administration of autoantigens, disease-suppressive fragments of said autoantigens and analogs thereof as an effective method for treating T-cell mediated autoimmune diseases.

Weiner et al., U.S. patent application Ser. No. 487,732, filed Mar. 2, 1990 now abandoned, discloses synergists (enhancers) for use with oral administration of autoantigens, disease suppressive fragments and analogs thereof as effective treatments for T-cell mediated autoimmune diseases.

Weiner et al., U.S. patent application Ser. No. 551,632 filed Jul. 10, 1990 now abandoned, a continuation-in-part of U.S. patent application Ser. No. 379,778, filed Jul. 14, 1989 now abandoned, discloses methods of preventing or treating uveoretinitis in mammals by oral administration of purified S antigen, Interphotoreceptor Retinoid Binding Protein (IRBP) antigen or disease suppressive fragments thereof.

Nagler-Anderson, et al., (*Proc. Natl. Acad. Sci. (USA)* 83: 7443-7446, 1986), describe the oral administration of collagen to suppress collagen-induced arthritis in a mouse model.

However, the above-mentioned references do not disclose the use of antigens to suppress the mammalian graft rejection mechanism because it has not been shown that the principle of oral administration of transplantation antigens could prevent allograft rejection.

The present invention proposes the clinical administration to mammalian graft recipients of alloantigens via oral and aerosol routes to induce a tolerance to foreign tissue grafts. The invention will be primarily useful in the field of organ transplantation including bone marrow. Although previous studies have shown that alloantigens injected intravenously to recipients can prolong the survival of renal transplants (*Transplantation* 39:56, 1985; *J. Immunol.* 121:1480, 1978; *J. Exp. Med.* 149:1042, 1979), no disclosure or suggestion of introducing these antigens orally or in an aerosol form was made therein.

It is, therefore, an object of the present invention to provide agents and methods for suppressing the detrimental immune response in mammals to the grafting or transplantation of foreign (or "non-self") tissues and organs.

Another object of the present invention is to provide pharmaceutical formulations and preparations that may be administered to mammals to suppress the immune rejection of surgically transplanted tissues.

A still further object of the invention is to provide synthetic compositions and pharmaceutical formulations that may be administered to mammals via the oral or aerosol route to suppress the mammalian immune response to the presence of transplanted tissue or organs.

These and other objects of the present invention will become apparent to those of ordinary skill in the art in light of the following.

SUMMARY OF THE INVENTION

It has now been unexpectedly discovered that compositions comprising specific antigenic agents, including by way of non-limiting example allogeneic spleen tissue and cultured lymphocytes and specific Major Histocompatibility Complex (MHC) antigens can be administered to mammals via the oral or aerosol route to suppress the mammalian immune response to surgically transplanted "non-self" organs or tissues. Because the effect is dependent upon MHC molecules present on the surface of spleen cells, which differ between the tissue donor and the recipient, administration of these antigens alone is expected to be effective.

Orally administered allogeneic splenocytes can suppress the immune response of a host mammal which normally occurs shortly after transplant surgery against surgically transplanted "non-self" tissue in an antigen-specific manner. It has also been found that oral ingestion of allogeneic spleen tissue preparations depresses the delayed type hypersensitivity reaction and mixed lymphocyte reaction in mammals. Compositions and pharmaceutical formulations for oral administration of allogeneic splenocytes may be prepared from natural allogeneic tissue. For administration to humans such compositions comprise synthetic derivatives of antigens i.e., peptide fragments of MHC antigens.

In practicing the method of the present invention, pharmaceutical formulations containing synthetic antigens or natural allogeneic splenic or lymphocyte tissue or cell derivatives are prepared and orally administered to mammalian subjects some time prior to organ or tissue transplant surgery.

Additionally, an aerosol delivery system can be prepared with essentially the dosages of splenocyte derivatives or MHC antigens as above and a pharmaceutically suitable carrier or diluent. The aerosol formulations can also be administered sometime prior to transplant surgery via the aerosol route. These and other improvements will be described in the following descriptions, drawings and appended claims.

BRIEF DESCRIPTION-OF THE DRAWINGS

FIG. 1 is a graph showing the effect of feeding allogeneic splenocytes on the mixed lymphocyte reaction (MLR).

FIG. 2 is a graph showing the effect of feeding allogeneic splenocyte lysates on the mixed lymphocyte reaction.

FIG. 3 is a graph showing the kinetics of oral tolerance to alloantigens.

FIG. 4 is a graph depicting the effect of feeding syngeneic or allogeneic splenocytes on delayed type hypersensitivity (DTH) reactions.

FIGS. 5A and 5B are a series of immunofluorescence analyses (histograms) showing the effect of feeding allogeneic splenocytes on lymphocyte composition.

FIG. 6 is a graph showing the survival of cardiac allografts in control rats, LEW rats fed syngeneic splenocytes, LEW rats fed third party (WF) splenocytes or LEW rats fed allogeneic splenocytes.

FIG. 7 is a graph showing the effects of feeding splenocytes on the MLR of skin graft recipients compared to control (non-fed) skin graft recipients.

DETAILED DESCRIPTION OF THE INVENTION

The contents of all patent applications, patents and literature references referred to in this specification are hereby incorporated by reference in their entirety.

The present invention addresses the need for an alternate to existing methods for suppressing the immune response directed-against foreign tissue transplants, as for example, post-transplant surgery. In addition, the methods of the present invention provide for prolonged survival of organ and tissue allogeneic grafts (i.e. transplants from individuals of the same species) in a mammal in need of such treatment.

Thus, the present invention provides means whereby the rejection of tissue allografts can be prevented, thus prolonging the survival of transplanted tissue and organs.

It has now been unexpectedly discovered that oral administration of allogeneic splenocytes or synthetic MHC antigens (or immune suppressive fragments or analogs thereof) is effective for suppressing the in vitro mixed lymphocyte reaction which is a model system for the graft rejection response in post-transplant mammalian recipients.

Without wishing to be bound to any particular theory of operation or mechanism of action for the invention it is believed that the oral administration of allogeneic splenocytes or derivatives of MHC antigens pursuant to the present invention affects the immunological mechanisms of graft rejection, i.e. the activation of helper T-cells is decreased by the induction of specific suppressor T-cells.

In the following discussions the following terms shall have the meaning ascribed to them below.

"Oral administration" shall mean both oral administration and enteral administration (delivery directly into the stomach).

"Mammal" shall mean any organism having an immune system and therefore susceptible to allogeneic graft rejection.

"Aerosol" refers to finely divided solid or liquid particles that may be created using a pressurized system such as a nebulizer. The liquid or solid source material contains MHC antigens and/or disease suppressive fragments and analogs thereof as defined herein.

The "aerosol route" of administration means delivery of an aerosol formulation to a host via the nasal or oral airway.

"Major Histocompatibility Complex" (MHC) is defined as a complex series of mammalian cell surface proteins. The MHC plays a central role in many aspects of immunity both in presenting histocompatibility (or transplantation) antigens and in regulating the immune response against conventional (foreign) antigens. There are two types of MHC protein molecules, Class I and Class II. Class I MHC proteins are present on virtually all tissues and Class II MHC proteins are present on the surface of activated T-cells, macrophages and other immune system cells. The human MHC genes (the HLA genetic locus) are located on human chromosome 6, the mouse MHC genes are located in the H-2 genetic locus on mouse chromosome 17 the analogous rat MHC genes are referred to as RTI.

"Class I MHC antigens" are defined as membrane glycoproteins present on the surface of all nucleated cells and play a key role in antigen recognition by CD8+ cytotoxic T-cells.

"Class II MHC molecules" are membrane glycoproteins that form part of the MHC and are most important in the initiation of immune responses. Class II MHC molecules are found mainly on cells of the immune system including B-cells, macrophages, brain astrocytes, epidermal Langerhan's cells, dendritic cells, thymic epithelium and helper T-cells. Class II MHC molecules are involved in regulating the immune response during tissue graft rejection, stimulation of antibody production, graft-versus-host reactions and in the recognition of "self" (or autologous) antigens, among other phenomena.

"MHC antigens" are defined herein as Class I and/or Class II MHC antigens. MHC antigens of the present invention include both Class I and Class II, either alone or in combination.

"Allogeneic tissue extracts" are defined as splenocyte, splenic tissue or cultured lymphocyte extracts obtained from an allogeneic transplant donor and prepared as described below.

"Immune suppressive fragments" means any peptide or polypeptide containing partial amino acid sequences or moieties of analogs of the relevant MHC antigens possessing the ability to induce suppression of the hosts immune response against organ or tissue allogeneic grafts. Such fragments need not possess the alloantigenic properties of the entire MHC molecule.

"Analog" of immune suppressive fragments refers to compounds that are structurally related to suppressive fragments of MHC antigens thereof which possess the same biologic activity, i.e., the ability to suppress a mammalian hosts response against a transplanted organ or tissue. The term includes peptides having amino acid sequences which differ from the amino acid sequence of the relevant MHC antigens of the potential graft recipient by one or more amino acid residues.

Disease suppressive fragments and analogs for use in the present invention can be synthesized using well known solid phase synthesis techniques (Merrifield, R. B. *Fed. Proc. Am. Soc. Ex. Biol.* 21: 412, 1962 and *J. Am. Chem. Soc.* 85: 2149, 1963; Mitchell, A. R. et al., *J. Am. Chem. Soc.* 98: 7357, 1976; Tam, J. et al., *J. Am. Chem. Soc.* 105: 6442, 1983). Analogues can be constructed by identifying an equivalent amino acid sequence and using the peptide synthesis techniques disclosed above.

Analogues can be provided using the known amino acid sequence of MHC antigens as disclosed in *Immunogenetics* 29:231-234, 1989.

Disease-suppressive analogs and fragments can also be obtained using recombinant DNA techniques that are well-known in the art.

Disease suppressive fragments of MHC antigens and analogs thereof can be identified using routine experimentation using suitable in vivo systems such as those of Examples 1-4 below.

T-lymphocytes can be obtained from a potential allograft donor using methods well known in the art and cultured as described in *Transplantation* 41:549, 1986 and *Transplantation* 48:639, 1989 and administered to a mammal about to undergo or having undergone (as described below) an organ or tissue allograft.

Extracts (or lysates) of splenic tissue or cultured lymphocytes can be prepared using techniques well known in the art such as those described in Example 1 below.

In accordance with the present invention, conventional tissue typing, well-known in the art and routinely conducted on all transplant donors and recipients, is performed on a potential transplant donor to determine the MEC phenotype of the donor tissue or organ. Synthetic MHC antigens, disease suppressive fragments or their analogs can then be synthesized using the techniques described above. These antigens and/or fragments may be administered to mammals, especially humans, who are to receive a transplant, or to patients that have already received transplanted "non-self" tissue. The methods and compositions of the present invention may be used to treat mammals that have previously received "non-self" organ or tissue trans-

plants and are beginning to display the initial symptoms of allograft rejection (such as fever, tenderness of the transplanted organ or loss of function thereof). The method and compositions of the invention are useful to preserve the organ or tissue and damp down or shut off that portion of the immune response of the recipient that is directed against the transplanted tissue or organ. To be effective the compositions and methods of the present invention must be administered before total rejection occurs.

Pursuant to the present invention, MHC antigens or transplantation rejection suppressive fragments or their analogs are ingested by a mammal that is to receive, or has already received a "non-self" organ or tissue transplant via the oral or enteral route, in an amount of between about 0.1 mg per kg body weight and about 10 mg per kg of body weight per day. The pharmaceutical compositions of the invention may be administered as a single dose or in multiple dose form via the oral or enteral route. Preferably, the is administered in an amount between about 1 mg and about 5 mg per kg body weight of said mammal per day. The exact amount to be administered will vary depending on the severity and stage of a patient's disease and the physical condition of the patient.

When administering splenic cells, cultured lymphocytes or extracts thereof, between about 10^6 and about 10^9 cell equivalents per kg body weight per day may be administered in single or divided doses.

The timing of such treatments shall be such that, if possible, the pharmaceutical formulations or dosage forms of the present invention are administered between about 7 and about 14 days before the transplantation is performed. The treatment is preferably continued for at least about 6 months after the transplanted organ or tissue has been introduced into the host (recipient) organism and may be continued indefinitely if necessary or desirable.

In addition, if a transplant recipient (either already receiving the compositions of the invention or not) begins to manifest symptoms of rejection, the pharmaceutical formulations of the present invention may be administered in increased amounts and/or frequency.

The present invention also is directed to oral dosage forms and pharmaceutical formulations for administration to mammals in order to prolong the survival of or suppress the rejection of a transplanted organ or tissue. It will be understood that any statistically significant prolongation in graft survival pursuant to the treatment of the present invention is within the scope of the invention.

The oral pharmaceutical formulations of the present invention may also contain inert constituents including pharmaceutically acceptable carriers, diluents, fillers, solubilizing or emulsifying agents and salts of the type that are well-known in the art. For example, tablets and caplets may be formulated in accordance with conventional procedures employing solid carriers, such as starch and bentonite, that are well-known in the art. Examples of solid carriers include bentonite, silica, dextrose and other commonly used carriers. Further non-limiting examples of carriers and diluents which may be used in the formulations of the present invention include saline and any physiologically buffered saline solution such as phosphate buffered saline, pH 7-8 and water.

Capsules employed in the present invention may be made from any pharmaceutically acceptable material such as gelatin or cellulose derivatives. The active biological materials of the invention may be administered in the form of sustained release oral delivery systems and/or enteric coated oral dosage forms such as those described in U.S. Pat. No.

4,704,292 issued Nov. 3, 1987, U.S. Pat. No. 4,309,404 issued Jan. 5, 1982 and U.S. Pat. No. 4,309,406 issued Jan. 5, 1982.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual dose of each dosage form need not in itself constitute an effective amount for suppressing graft rejection since the necessary effective amount can be reached by administration of a plurality of dosage units.

The preferred route of administration of the dosage forms of the present invention is orally or enterally. Preferred oral or enteral pharmaceutical formulations or dosage forms may comprise for example, between about 70 mg and about 500 mg of MHC antigens, disease suppressive fragments or analogs thereof or between about 10^7 - 10^{10} cell equivalents when using allogenic cells or extracts thereof.

In an alternative embodiment of the present invention the pharmaceutical formulations of the present invention are administered to mammals in aerosol form. It is anticipated that smaller quantities of the allogeneic tissue extracts or MHC antigens, disease suppressive fragments or their analogs will be required to achieve suppression of graft rejection when using the aerosol form of administration. This has been found to be the case in treating experimental allergic encephalomyelitis (EAE) with myelin basic protein (MBP), and also in treating adjuvant arthritis with collagen as disclosed in the co-pending U.S. patent application of Weiner et al. Ser. No. 454,806 filed Dec. 20, 1989 now abandoned. The quantity of MHC antigens, disease suppressive fragments or the analogs of such materials which may be administered in an aerosol dosage form would be between about 0.01 mg and 10 mg per kg body weight of a mammal per day. The aerosol dosage forms of the present invention may be administered to a patient via the aerosol route in a single dosage form or multiple dosage forms. The exact amount to be administered will vary depending on the state and severity of a patient's disease, the activity of the patient's immune system and the physical condition of the patient.

When administering splenic cells, cultured lymphocytes or extracts thereof, between about 10^5 and about 10^9 cell equivalents per kg body weight per day may be administered in single or divided doses in an aerosol form.

The aerosol pharmaceutical formulations of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are well-known in the art. Specific non-limiting examples of the carriers and/or diluents that are useful in the aerosol pharmaceutical formulations of the present invention include water, normal saline and physiologically-acceptable buffered saline solutions such as phosphate buffered saline solutions, pH 7.0-8.0.

Examples of useful solubilizing and emulsifying agents are physiologically balanced salt solutions, phosphate buffered saline and isotonic saline. The salts that may be employed in preparing the aerosol dosage forms of the invention include the pharmaceutically acceptable salts of sodium and potassium.

The route of administration of allogeneic spleen cells, cultured lymphocytes extracts thereof or MHC antigen or disease suppressive fragments or their analogs according to this alternate embodiment of the present invention is in an aerosol or inhaled form. The aerosol compositions of the present invention can be administered as a dry powder or in an aqueous solution. Preferred aerosol pharmaceutical for-

mulations may comprise, for example, a physiologically-acceptable buffered saline solution containing between about 7 mg and about 700 mg of the compositions of the present invention, disease suppressive fragments or analogs thereof.

Dry aerosol in the form of finely divided solid particles of tissue extracts from spleen cells, MHC antigens disease suppressive fragments or analogs thereof that are not dissolved or suspended in a liquid are also useful in the practice of the present invention. The compositions of the present invention may be in the form of dusting powders and comprise finely divided particles having an average particle size of between about 1 and 5 microns, preferably between 2 and 3 microns. Finely divided particles may be prepared by pulverization and screen filtration using conventional techniques that are well known to those skilled in the art. The particles may be administered by inhaling a predetermined quantity of the finely divided material, which can be in the form of a dry atomized powder.

The pharmaceutical formulations of the present invention may be administered via the aerosol route by means of a nebulizer, as an example those described in U.S. Pat. Nos. 4,624,251 issued Nov. 25, 1986; 3,703,173 issued Nov. 21, 1972; 3,561,444 issued Feb. 9, 1971 and 4,635,627 issued Jan. 13, 1971. The aerosol material is inhaled by the subject to be treated.

Other systems of aerosol delivery, including for example the pressurized metered dose inhaler (MDI) and the dry powder inhaler as disclosed in Newman, S. P. in *Aerosols and the Lung*, Clarke, S. W. and Davia, D. eds. pp. 197-224, Butterworths, London, England, 1984 can be used in conjunction with the method of the present invention.

Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, Mass.), Schering Corp. (Kenilworth, N.J.) and American Pharmoseal Co., (Valencia, Calif.).

In accordance with the present invention, experiments were performed in which the effects of oral administration of allogeneic splenocytes to Lewis rats were studied, with particular attention being given to the effects on the immune response of the transplant recipient. To this end, the in vitro mixed lymphocyte response (MLR), the delayed type hypersensitivity (DTH) reaction, and the in vivo accelerated cardiac allograft rejection techniques were utilized. In each case, the oral administration (to the recipient of a "non-self" tissue transplant) of splenocyte cells from a donor animal resulted in suppression of these T-cell mediated immune reactions. As T-cells have been implicated as the major mediators of allograft rejection, the results of these tests establish the practical efficacy of the methods and pharmaceutical formulations of the present invention.

The present invention is illustrated in specific working examples presented below which are intended to illustrate the present invention without limiting the scope thereof.

EXAMPLE 1: PREPARATION OF MATERIALS AND TEST SUBJECTS

1. Subject Mammals

The test population was comprised of male rats of the Lewis (LEW), Wistar Furth (WF) and Brown Norway (BN) variety (obtained from Harlan Sprague Dawley Inc., Indianapolis, Ind.). The rats in the experiments described below were approximately 8-10 weeks old, and were bred under careful observation.

2. Preparation of Splenocytes For Oral Administration

Fresh splenic tissue was obtained from syngeneic (same species, same strain) or allogeneic (same species, different strain) animals shortly prior to oral administration. Single cell splenocyte suspensions were prepared by mashing the fresh spleen through a standard stainless steel mesh (2 inches by 2 inches). Red blood cells were specifically lysed with Tris-ammonium chloride buffer according to standard procedures well known in the art, washed twice with Hank's Balanced Salt Solution (HBSS) and resuspended into various concentrations as described below before use.

3. Preparation of Splenocyte Lysate

Splenocytes prepared as in the above method were lysed by repetitive freeze-thawing in the following manner:

- (a) Cells were quick frozen at -70° C. for 30 minutes;
 - (b) Quick frozen splenocytes were then thawed at 37° C.;
 - (c) This freeze-thaw cycle was repeated one more time.
- The resulting materials were used for oral administration.

EXAMPLE 2: ORAL ADMINISTRATION OF PREPARED SPLENOCYTE SUSPENSION

A one milliliter dose of the cell suspension as prepared in Example 1, was orally introduced to each test rat with a syringe having a ball-tipped feeding needle (Thomas Scientific, Swedesboro, N.J.).

The following laboratory immunological and pathological procedures were conducted on the lymphatic organs of the test rats.

EXAMPLE 3: MIXED LYMPHOCYTE REACTION

Cervical lymph nodes were taken from the responder (LEW) and the stimulator (WF or BN) rats. The excised nodes were then pressed through stainless steel mesh as above and suspended in Phosphate Buffered Saline.

The isolated lymph node cells were then washed twice and resuspended into RPMI 1640 medium, containing 10% fetal calf serum (FCS), 1% penicillin and streptomycin (Microbiological Associates, Walkersville, Mass.) 2×10^{-5} M 2-mercaptoethanol, and 5 mM HEPES, at a concentration of 6×10^6 cells/ml. Responder cells were seeded into a 96-well flat-bottomed culture plates (Costar Cambridge, Mass.) at 50 microliters per well, with or without irradiated stimulator cells (3000 Rads gamma irradiated using a Shepherd irradiator, Model 143-45 and a Cesium-137 source) of the same volume.

The treated cells were then cultured at 37° C. with 5% CO_2 for four days before they were pulsed for 6 hours with ^3H -thymidine (1 microCi/well, obtained from NEN Dupont, Boston, Mass.). Cell proliferation was monitored by incorporation of ^3H -thymidine measured by a Beckman liquid scintillation counter.

SUPPRESSOR ASSAY

Obtained lymph node cells were irradiated (1000 Rads of gamma radiation) and added to a test MLR at concentrations varying from 5 to 20% of total cells per well (experimental wells). Control wells were set up with no modulators while background wells had only responder cells. These cultures were incubated at 37° C. and in 5% CO_2 for 96 hours. Proliferation was assayed by pulsing the plates with 1 microCi/well ^3H -thymidine for the last 6 hours of culture. The plates were then harvested as described above.

DELAYED TYPE HYPERSENSITIVITY DTH REACTIONS

Rats of each group were immunized subcutaneously in the footpad with 10 million gamma irradiated (3000 RAD) allogeneic splenocytes. Ten days later, they were injected again with the same dosage in the ear lobe. The responses were determined as the changes in the ear thickness before and 48 hours after the challenge.

CELL TYPING

The phenotypes of the extracted lymphocytes were tested by indirect immunofluorescent staining and with a fluorescence-activated cell sorter (FACS). The lymph cells were first incubated for 1 hour with primary monoclonal antibodies against the cell surface markers CD4 or CD8, or mouse immunoglobulin (Organon-Teknica, Westchester, Pa.) and washed twice with PBS containing 0.02% sodium azide. They were then further incubated with FITC-conjugated goat-anti-mouse IgG (1:40) (Organon Teknica) in the dark for 30 minutes and in the presence of 15% autologous normal rat serum. The cells were thoroughly washed and fixed with 1% formaldehyde before testing.

Additionally, surgical transplant methods of the type described in the following example were performed.

EXAMPLE 4: CARDIAC ALLOGRAFT

LEW rats were subjected to surgical transplant procedures. An accelerated rejection model was used wherein LEW strain rats were pre-sensitized with BN strain full-thickness skin grafts seven days before the cardiac allograft, with and without oral ingestion of splenocyte preparations.

Seven days later, a (LEWxBN)F1 strain test vascularized cardiac allograft was performed on each pre-treated rat. The cardiac grafts were anastomosed to the infra-renal abdominal aorta. Rejection was defined as complete cessation of heart beat as determined by daily palpation of the recipient's flank.

The above-described methods were used to obtain the following results:

I. SUPPRESSION OF THE MIXED LYMPHOCYTE REACTION (MLR) BY ORAL ADMINISTRATION OF ALLOGENEIC SPLENOCYTE PREPARATIONS

Splenocytes from WF rats were freshly prepared and were administered orally to LEW rats two, five or ten times over a 1-2 week period.

The individual dosages were 50 million cells per oral administration.

Seven days following the last oral administration, lymph nodes were taken from both a control group and those given oral splenocytes for MLR studies using WF or BN stimulators. As shown in FIG. 1, LEW rats which had ingested allogeneic splenocytes showed significantly reduced reaction against the lymphocytes from the WF strain. This phenomenon was observed in all three feeding protocols (i.e., 2, 5 or 10 times). However, only the group that received ten feedings showed suppression against the BN strain, the third party control.

These results indicate that limited ingestion of allogeneic splenocyte preparations induced antigen specific suppression of the MLR.

II. COMPARISON OF SUPPRESSION OF MLR BY ORAL ADMINISTRATION OF ALLOGENEIC VERSUS SYNGENEIC SPLENOCYTE PREPARATIONS

A dose response study was subsequently conducted to determine the effect of feeding syngeneic versus allogeneic cells. LEW rats were fed twice with 1, 5, 25 or 50 million splenocytes from either LEW or WF strains. The results are set forth in Table I below.

TABLE I

The Effect of Feeding Syngeneic and Allogeneic Splenocytes on MLR			
Strains used for feeding	Dosage cells/feeding	MLR delta/CPM	Relative Response (%)
LEW	—	115015 ± 7707	100
	1 × 10 ⁶	128520 ± 8338	112
	5 × 10 ⁶	54391 ± 10988	47
	25 × 10 ⁶	39088 ± 7294	34
	50 × 10 ⁶	81329 ± 8013	71
WF	1 × 10 ⁶	71135 ± 13721	62
	5 × 10 ⁶	79011 ± 14119	68
	25 × 10 ⁶	56196 ± 15254	49
	50 × 10 ⁶	73541 ± 11636	64

Feeding at the lowest dosage (1 million) of syngeneic cells did not induce suppression; all other doses, both syngeneic and allogeneic cells, show some suppression to varying degrees.

III. EFFECT OF INGESTED LYSATE OF ALLOGENEIC SPLENOCYTE PREPARATIONS ON MLR

The effect of ingested lysate alone on MLR was next studied to determine whether live splenocytes were required for the orally induced tolerance. Rats were given two separate oral doses of either live splenocytes or the corresponding lysate prepared by the repetitive freeze and thaw method (described above) and the effect of these treatments were compared. FIG. 2 shows that cell lysate alone was sufficient in suppressing the MLR, indicating that a subcellular fragment was involved in suppressing the cell-mediated immunity.

IV. KINETICS OF MLR SUPPRESSION BY ORAL ADMINISTRATION OF ALLOANTIGENS

The kinetics of the orally induced tolerance to alloantigen was studied by giving two oral doses of splenocytes to separate LEW rat groups, 14 days, 7 days, 3 days, and 1 day before the MLR was performed. As shown in FIG. 3, the groups which were given oral doses 1 day or 3 days before MLR was performed did not induce suppression. The groups with 7-day and 14-day intervals between the last oral ingestion and MLR showed dramatic reduction of proliferation in MLR, indicating that more than 4 days were required for the induction of oral unresponsiveness to alloantigens.

V. SUPPRESSION OF DTH RESPONSE AGAINST ALLOANTIGENS

In addition to the in vitro MLR, the effect of ingesting allogeneic splenocytes on the delayed type hypersensitivity (DTH) response, in vivo, in LEW rats was examined. LEW rats were orally administered 10 feedings of 50 million splenocytes from either syngeneic or allogeneic (WF) animals. After the last oral ingestion, the test for DTH was initiated with the animals being immunized subcutaneously in their foot pads. The same animals were injected again 10 days later in the ear lobes. The DTH was measured as the changes in the ear thickness before and 48 hours after the challenge. The results are shown in FIG. 4.

Approximately 50% decrease in DTH response to WF was observed in rats fed with cells of the same strain, but not

in those fed with syngeneic LEW splenocytes. The DTH response against BN was not affected by the pre-treatment, indicating that the DTH suppression was antigen specific. **VL ACTIVE SUPPRESSION IS INVOLVED IN MEDIATING DECREASED PROLIFERATION IN THE MLR**

In order to study the mechanism of inhibition of MLR proliferation in the fed animals, a suppressor cell assay was performed to determine if CD8+ suppressor cells were involved in mediating the observed effects. Lymphocytes from either control or pre-fed animals were irradiated with 1000 RADS of gamma radiation before being added to a primary MLR, serving as modulators.

Lewis rats (3/group) were pre-treated 10 times orally with varying dosages (as indicated in the Table) of WF splenocytes. One week later, their cervical lymph nodes were taken and the cells served as modulator after being irradiated 1000 Rad of gamma radiation. The primary LEW anti-WF and LEW anti-BN MLR and Con-A stimulation cultures were set up as described above. Modulator cells were added to the primary cultures at a 1/5 ratio. The results are set forth in Table II below.

TABLE II

SUPPRESSION OF PRIMARY MLR BY LYMPHOCYTES FROM ALLOGENEIC SPLENOCYTES FED RATS						
Source of modulator	anti-WF		anti-BN		Con A	
	CPM ($\times 10^{-3}$)	% Supp.	CPM ($\times 10^{-3}$)	% Supp.	CPM ($\times 10^{-3}$)	% Supp.
control	112 \pm 21		405 \pm 78		280 \pm 4.7	
fed 10 $\times 10^6$	104 \pm 11	7	464 \pm 15	0	276 \pm 6.6	1.4
fed 25 $\times 10^6$	106 \pm 17	5.3	334 \pm 120	17	277 \pm 34	1
fed 50 $\times 10^6$	84 \pm 15	25	443 \pm 17	0	305 \pm 3.9	0
fed 50 $\times 10^6$	1 \pm 0.9	99	84 \pm 7.1	79	199 \pm 12	29

The results in Table II show that adding 20% of modulators from pre-fed animals, but not from the control animals, suppressed the primary LEW-anti-WF MLR. This suggests that suppressor cells were induced after feeding and these in turn mediated suppression of the MLR.

VII. PHENOTYPE OF LYMPH NODE CELLS FROM FROM ANIMALS INGESTING SPLENOCYTES

Cervical lymphocytes from either control or fed animals were cultured with irradiated WF stimulators for 5 days, then sorted for CD4+ or CD8+ cells by indirect immunofluorescence staining. The results shown in FIG. 5 show that pre-feeding rats with allogeneic splenocytes resulted in an increase in CD8+ (suppressor T-cells) cells and a decrease in CD4+ (helper T-cells) cells when compared to controls.

VIII. ORAL ADMINISTRATION OF SPLENOCYTES PREVENTS ACCELERATED CARDIAC ALLOGRAFT REJECTION

To demonstrate the prevention of allograft rejection, an accelerated rejection transplantation model, as described above, was used. LEW rats were pre-sensitized with BN skin grafts 7 days before challenge with vascularized BN test cardiac allografts, to study the effects of feeding allogeneic donor splenocytes on test graft survival.

While unsensitized controls rejected their cardiac allografts on the 6th through the 8th day, all sensitized control animals hyperacutely rejected their cardiac allografts within 36 hours. Test animals fed 5-10 feedings of 50 million splenocytes, 7 days prior to the skin graft, or even on the day of the skin graft, exhibited increased test cardiac allograft survival, to 7.62 \pm 0.5 days.

These results show that feeding allogeneic splenocytes prevents sensitization and converts accelerated rejection into an acute form.

The specificity of this phenomenon was examined as described below.

Cardiac recipient LEW rats were either unfed (n=10), fed LEW (syngeneic) lymphocytes (n=8), fed BN splenocytes (but received a WF cardiac allograft, n=6) or were fed BN splenocytes (and received a BN cardiac allograft, n=8). All fed animals received 5-10 feedings of 50 $\times 10^6$ splenocytes. The results are shown in FIG. 6.

As can be seen in FIG. 6, only the rats which were fed allogeneic splenocytes showed cardiac allograft survival beyond day 3. LEW rats fed third party (BN) lymphocytes but receiving a WF graft did not demonstrate enhanced cardiac allograft survival, demonstrating the specificity of this reaction.

In a preliminary attempt to study the mechanism of graft prolongation, the MLR of cervical lymph node cells from control and fed sensitized LEW rats were examined at 48 hours after the cardiac transplant. The results are shown in FIG. 7.

There was a suppression of the MLR in the fed animals as compared to the control (FIG. 7). These data are consistent with the previous MLR findings in the naive animal model.

What is claimed is:

1. A method for suppressing the immune response of a recipient mammal to non-self tissue from a donor mammal comprising:

orally or enterally administering to said recipient mammal an agent in an amount effective for suppressing said immune response, said agent comprising a MHC antigen or a peptide fragment of a MHC antigen said MHC antigen being from the donor of said non-self tissue or syngeneic to the donor of said nonself tissue.

2. A method for suppressing allograft rejection in a mammal comprising orally or enterally administering to a mammal in need of such treatment an agent in an amount effective to suppress the immune response of said mammal directed against said allograft, wherein said agent comprises a MHC antigen or a peptide fragment of a MHC antigen said MHC antigen being from the donor of said allograft.

3. The method of claim 1 comprising orally or enterally administering said agent prior to the recipient mammal receiving said non-self tissue.

4. A method for suppressing the immune response of a recipient mammal to non-self tissue from a donor mammal comprising:

orally or enterally administering to said recipient mammal an agent in an amount effective for suppressing said immune response, said agent comprising a Class II MHC antigen or a peptide fragment of a Class II MHC antigen said MHC antigen being from said donor or syngeneic to said donor.

5. A method for suppressing allograft rejection in a mammal comprising orally or enterally administering to a mammal in need of such treatment an agent in an amount effective to suppress the immune response of said mammal directed against said allograft, wherein said agent comprises a Class II MHC antigen or a peptide fragment of a Class II MHC antigen said MHC antigen being from said donor or syngeneic to said donor.


6. The method of claim 4 comprising orally or enterally administering said agent prior to the recipient mammal receiving said non-self tissue.

EXHIBIT 23

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What's New

2005 KFOC Medal for Research Excellence

Dr. Anthony Jevnikar – 2005 Winner of the Medal for Research Excellence
Kidney Foundation honours Dr. Anthony M. Jevnikar

For his outstanding work in the field of kidney disease and transplantation, Dr. Anthony Jevnikar was awarded The Kidney Foundation of Canada's Medal for Research Excellence on October 14, 2005.

Unique to the Foundation, this award pays tribute to a Canadian resident recognized nationally and internationally for excellence in renal-related research.

Both a clinical and basic science researcher, Dr. Jevnikar has made significant contributions in the areas of renal injury and immunotherapy over the past 14 years. He is Director of Transplantation Nephrology at the London Health Sciences Center and Professor of Medicine, Immunology and Microbiology at The University of Western Ontario in London.

Dr. Jevnikar's primary research interest lies in the molecular mechanisms of renal transplant injury – a principal cause of premature graft failure. Following a transplant, the kidney's response to stress and inflammation includes the expression of certain molecules within the tubular epithelial cells, which regulate the nature and consequence of injury. Dr. Jevnikar's findings reveal that the susceptibility or resistance to injury of these cells directly

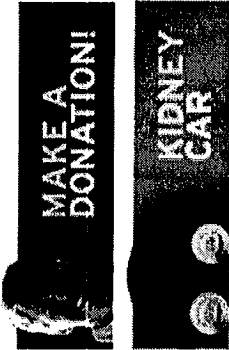


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influences the long-term function of the new organ. He believes that these findings, in combination with existing therapies, will contribute to more effective strategies to optimize survival of the transplanted organ.

He has also worked extensively to develop novel expression and drug delivery systems. He and his team, for example, are collaborating with agricultural scientists and molecular biologists to create transgenic plants that express human proteins for topical and oral delivery of proteins that modulate the body's immune response. Using genetically altered plants to create and deliver biotherapeutics may represent a unique strategy in the prevention and treatment of diseases such as Type I diabetes, inflammatory bowel disease and lupus, as well as organ rejection.

Dr. Jevnikar has published over 80 peer-reviewed papers in highly respected journals. He has trained and mentored medical residents, graduate students and post-doctoral fellows in his laboratory and has served on numerous local, national and international committees as an advisor in the area of transplantation. A past president of the Canadian Society of Transplantation, he has also participated on many grants panels, including the Canadian Institutes of Health Research (CIHR). With his multidisciplinary and innovative approach to research, Dr. Jevnikar is bridging the bench to bedside gap and setting the stage for future generations of clinician researchers in Canada.

Through the funding of research initiatives and related clinical education, The Kidney Foundation's Research Program supports investigators like Dr. Jevnikar in their quest to expand the boundaries of science and explore new treatment options that will improve the quality of life of Canadians.

The only national health charity serving the particular needs of people living with kidney disease, The Kidney Foundation of Canada funds research, provides educational and support services, advocates for access to high quality healthcare, and actively promotes kidney health and organ donation.

EXHIBIT 24



US 20080253991A1

(19) **United States**(12) **Patent Application Publication**
Jevnikar et al.(10) **Pub. No.: US 2008/0253991 A1**(43) **Pub. Date: Oct. 16, 2008**(54) **ANTI-T CELL AND AUTOANTIGEN
TREATMENT OF AUTOIMMUNE DISEASE****Publication Classification**(76) **Inventors:** Anthony Jevnikar, London (CA);
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Carmel, IN (US); Sean Michael
Russell, Indianapolis, IN (US);
Janna M. Armstrong, Indianapolis,
IN (US)(51) **Int. Cl.**
A61K 45/00 (2006.01)
A61K 39/395 (2006.01)
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C12N 15/00 (2006.01)
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A61P 37/00 (2006.01)
G01N 33/00 (2006.01)
C07K 14/00 (2006.01)
C12N 15/11 (2006.01)
A61K 38/28 (2006.01)

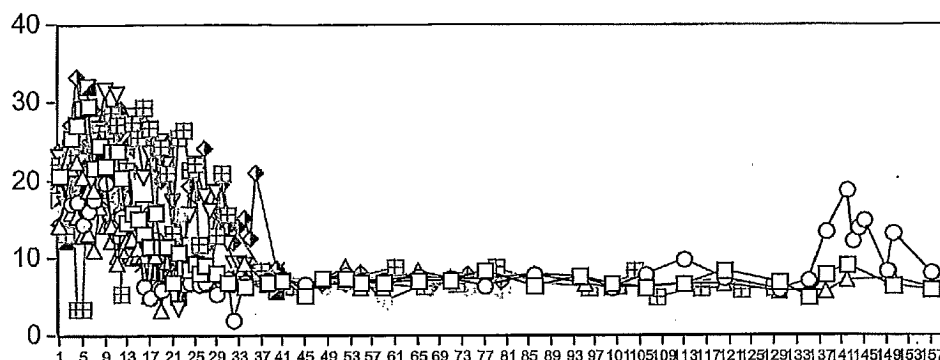
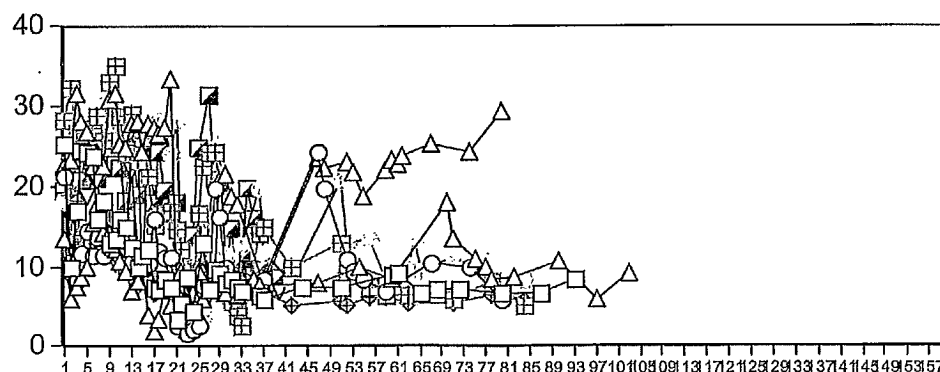
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CLEVELAND, OH 44114 (US)(52) **U.S. Cl.** 424/85.2; 424/154.1; 424/173.1;
514/12; 514/3; 536/23.5; 435/320.1; 530/351;
800/298; 436/63(21) **Appl. No.:** 11/815,359(22) **PCT Filed:** Feb. 6, 2006(86) **PCT No.:** PCT/CA06/00144

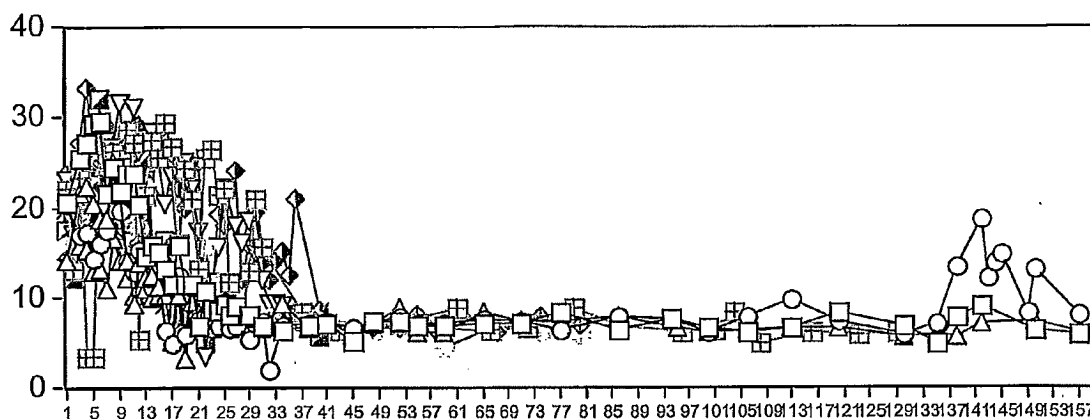
§ 371 (c)(1),

(2), (4) **Date:** Jun. 23, 2008**Related U.S. Application Data**(60) Provisional application No. 60/649,565, filed on Feb.
4, 2005.(57) **ABSTRACT**

The invention is directed to a new method for the treatment of new onset Type I diabetes in mammals or for the treatment of pre-Type I diabetic mammals where the method comprises administering (a) anti-T cell therapy to the mammal and administering (b) an autoantigen and optional mucosal antigen composition, wherein (a) and (b) are administered concurrently or sequentially Exemplified is a treatment using a mixture of anti-CD3 antibodies, a glutamic acid decarboxylase (GAD) autoantigen, and an immunoregulatory cytokine Canine GAD sequences are also disclosed.

GAD/IL4 feed mice**Control feed mice**

GAD/IL4 feed mice



Control feed mice

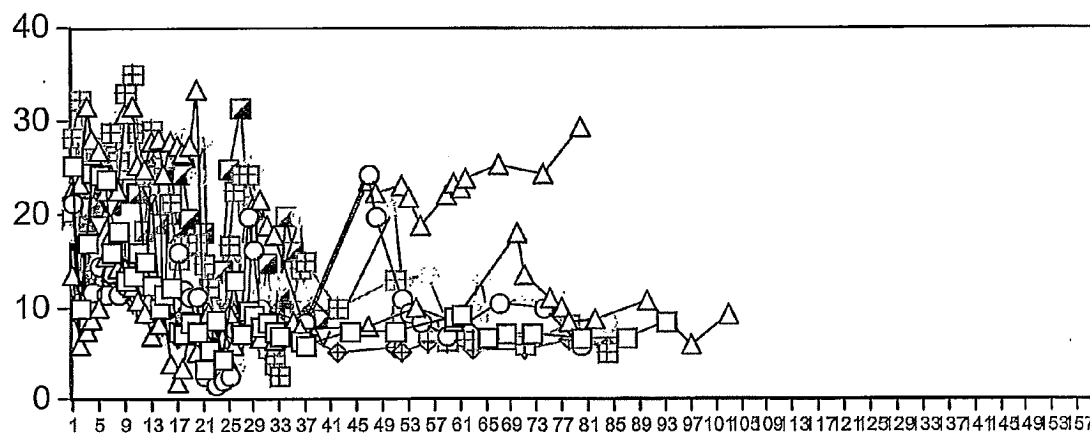


FIGURE 1

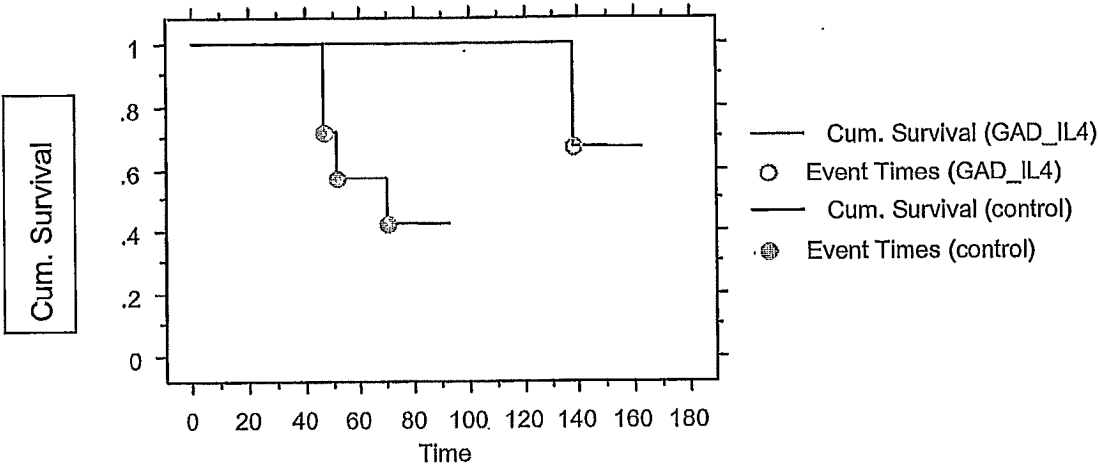


FIGURE 2

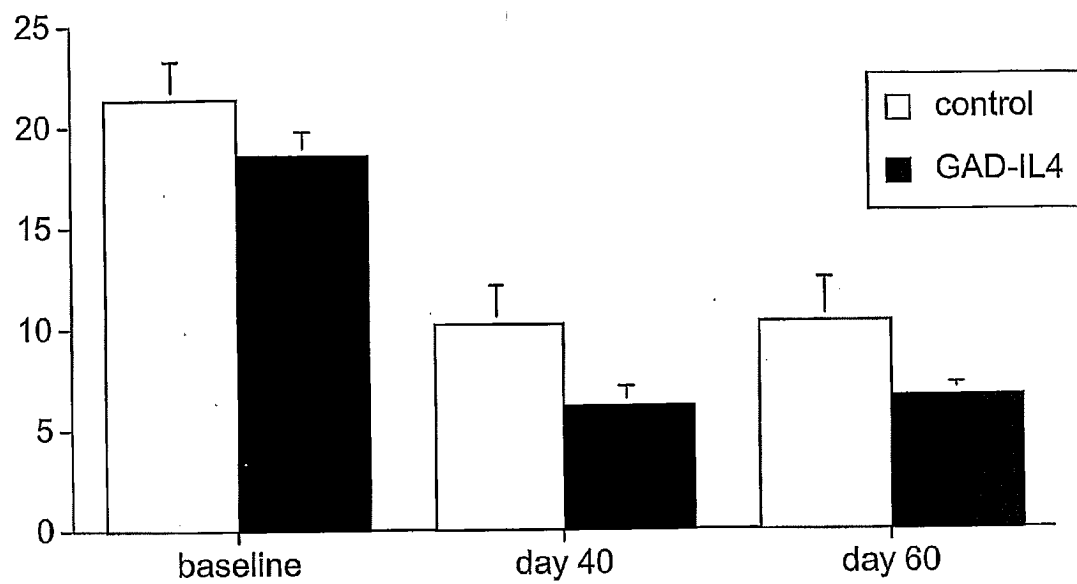
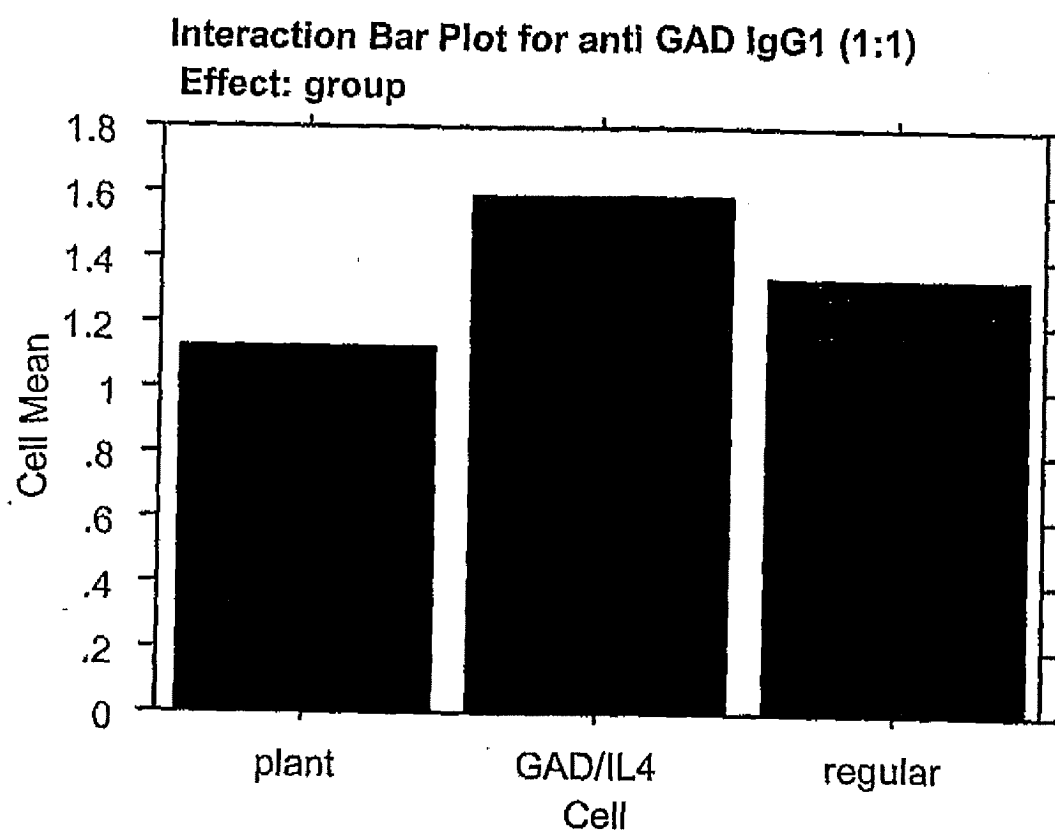
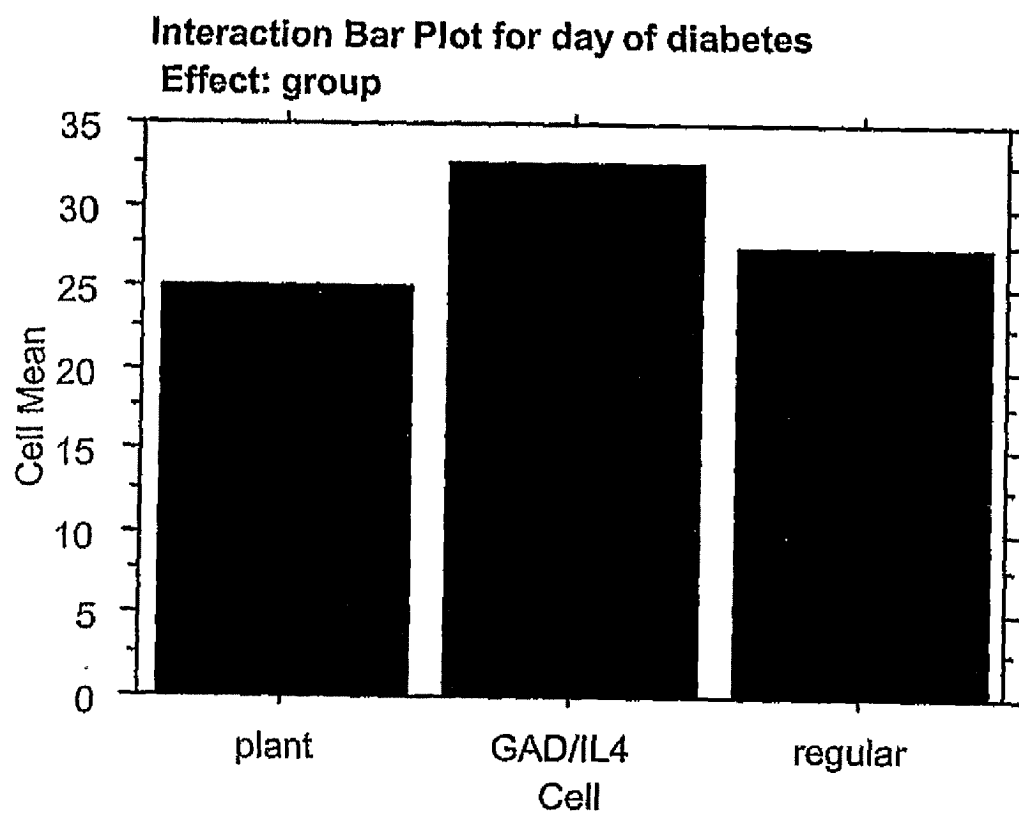
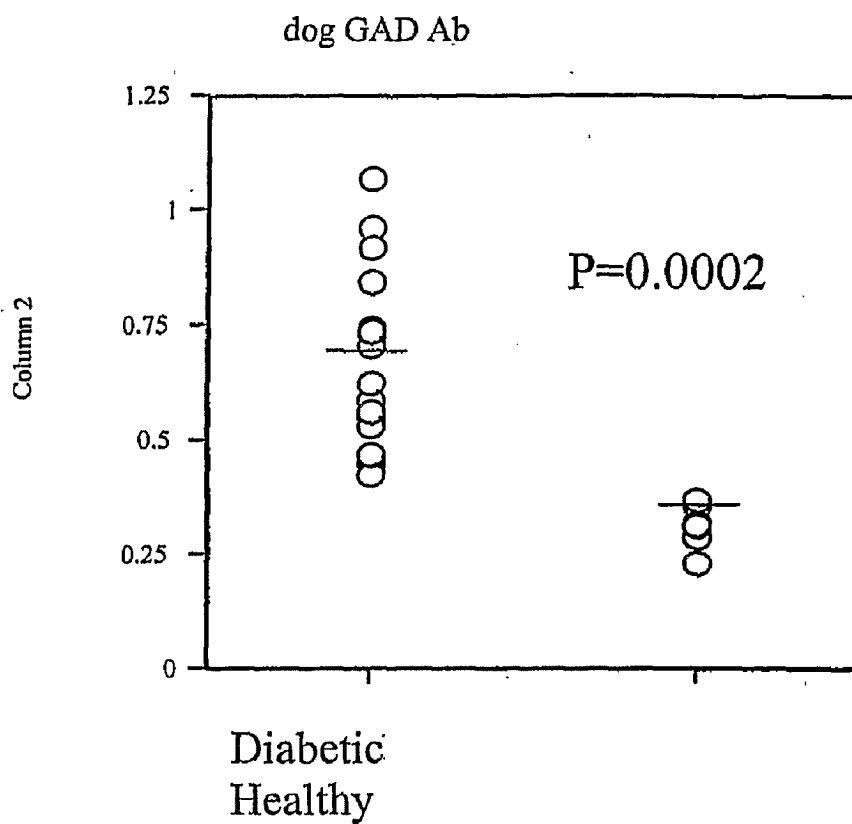


FIG. 3

**FIGURE 4**

**FIGURE 5**



Anti GAD65 antibodies in diabetic dogs

FIGURE 6

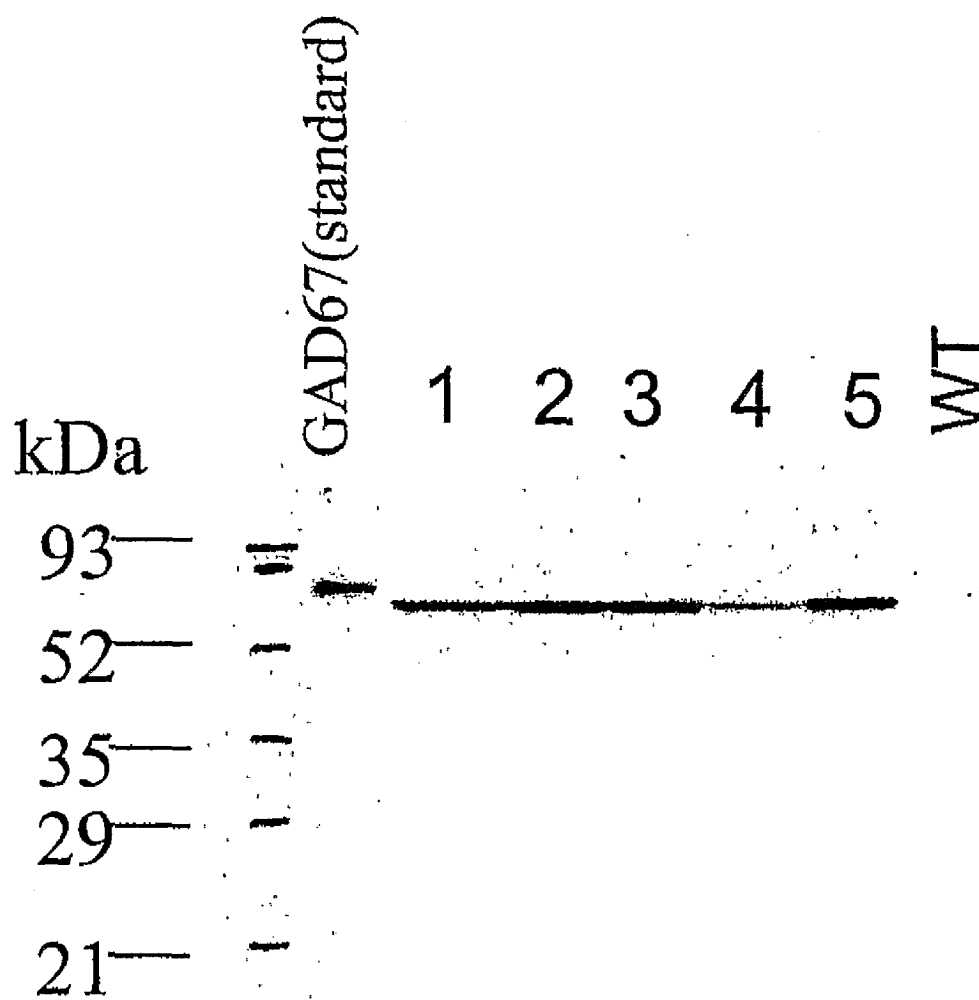
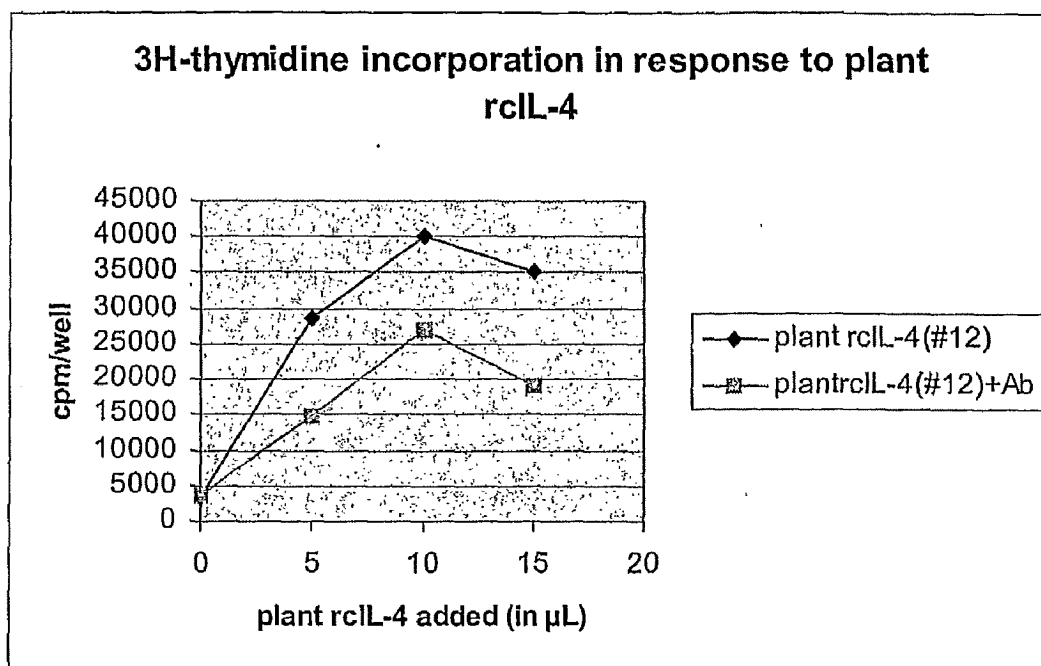
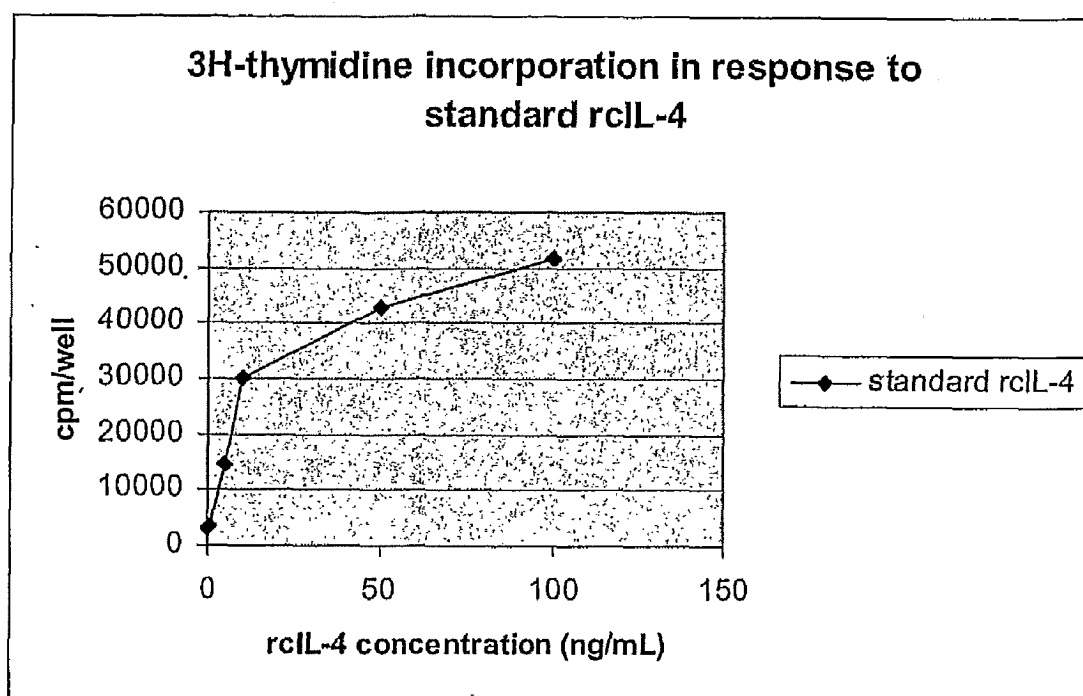


FIGURE 7

**FIGURE 8A**

**FIGURE 8B**

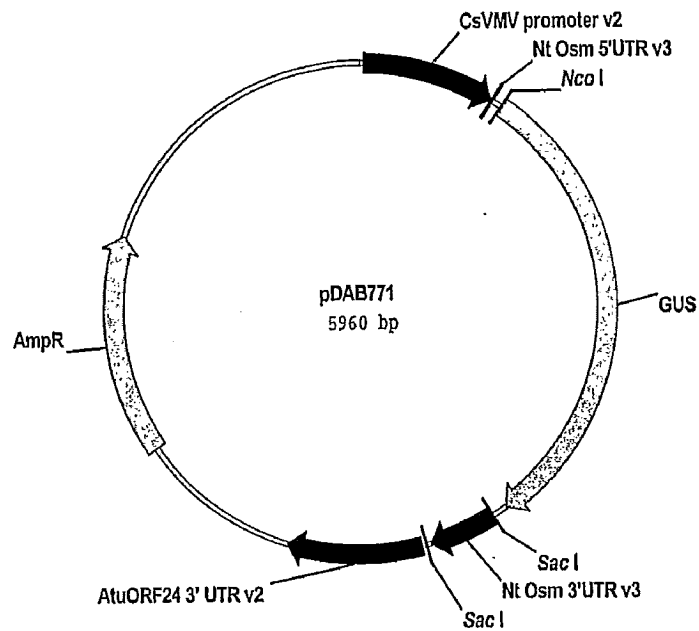


FIGURE 9A

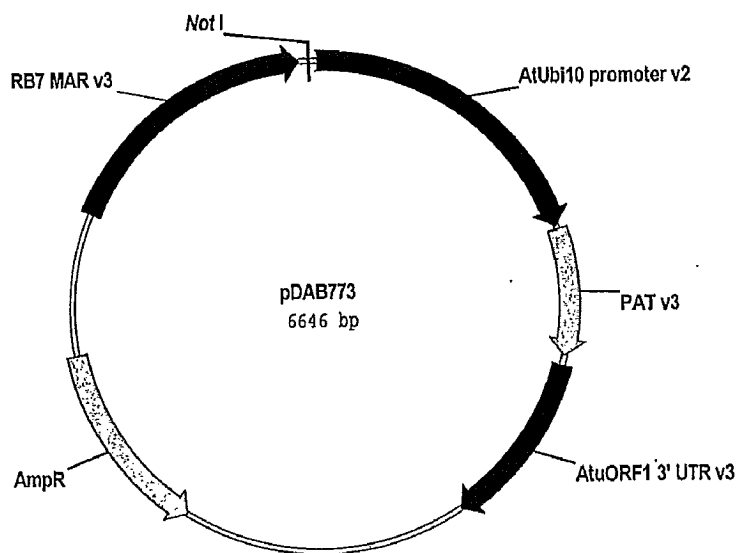


FIGURE 9B

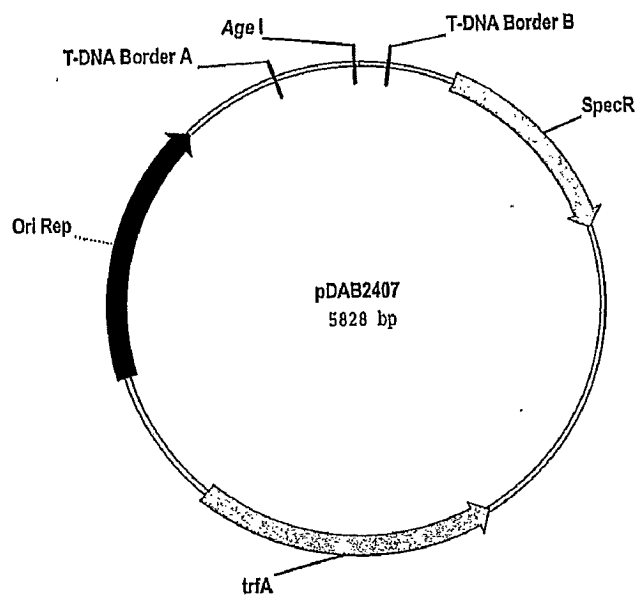


FIGURE 9C

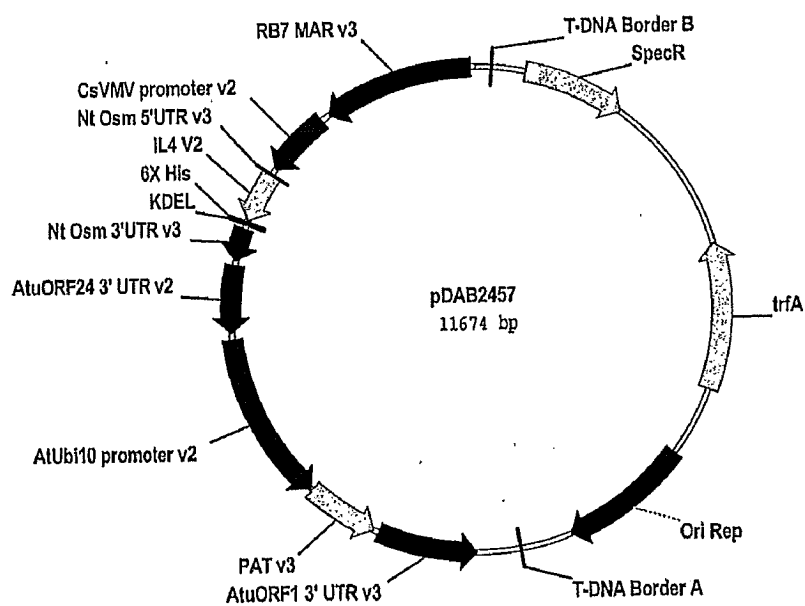


FIGURE 9D

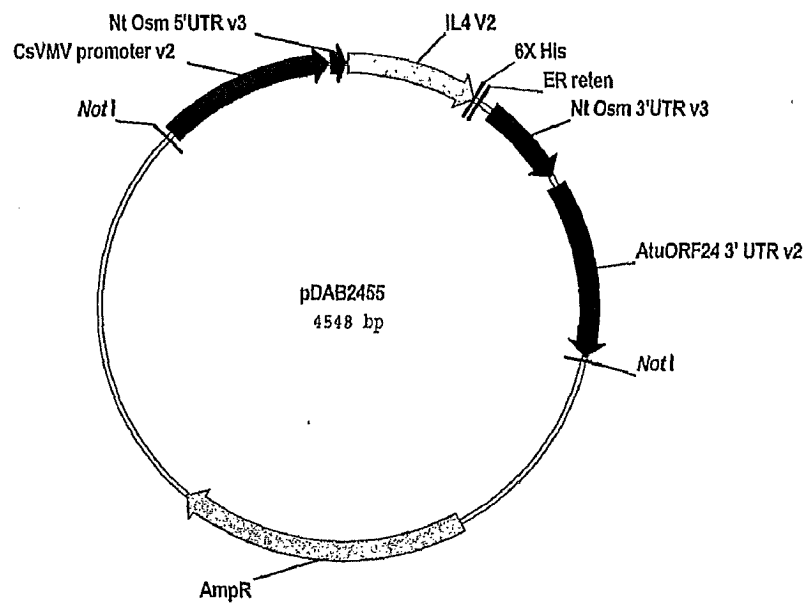


FIGURE 9E

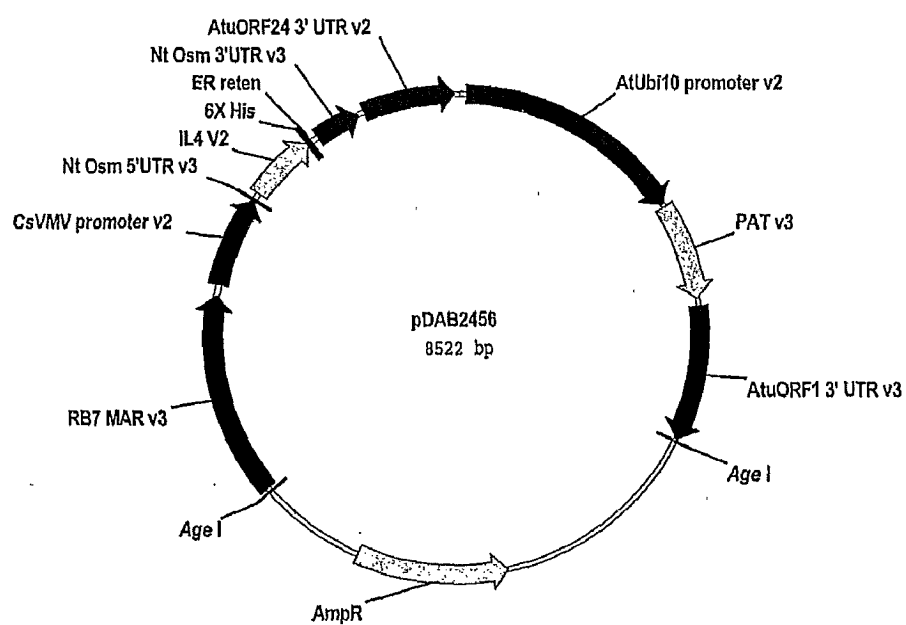


FIGURE 9F

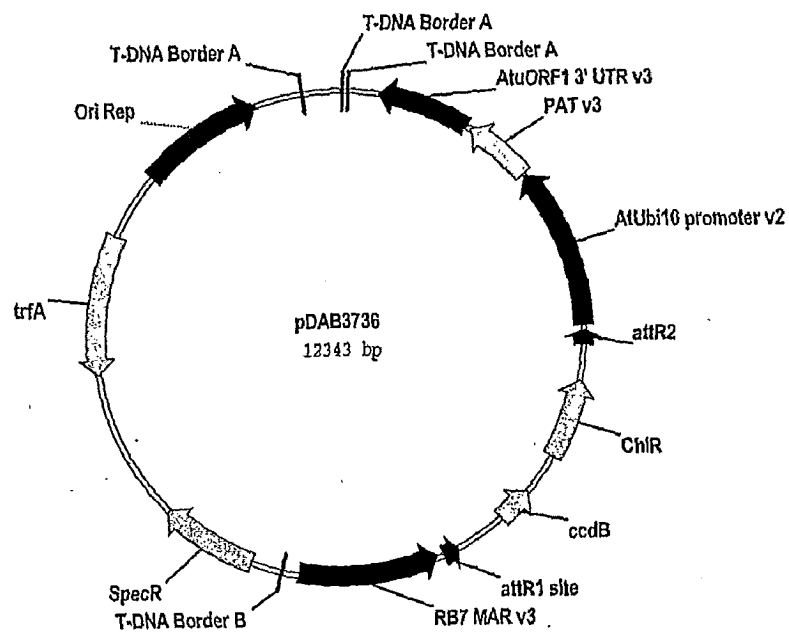


FIGURE 9G

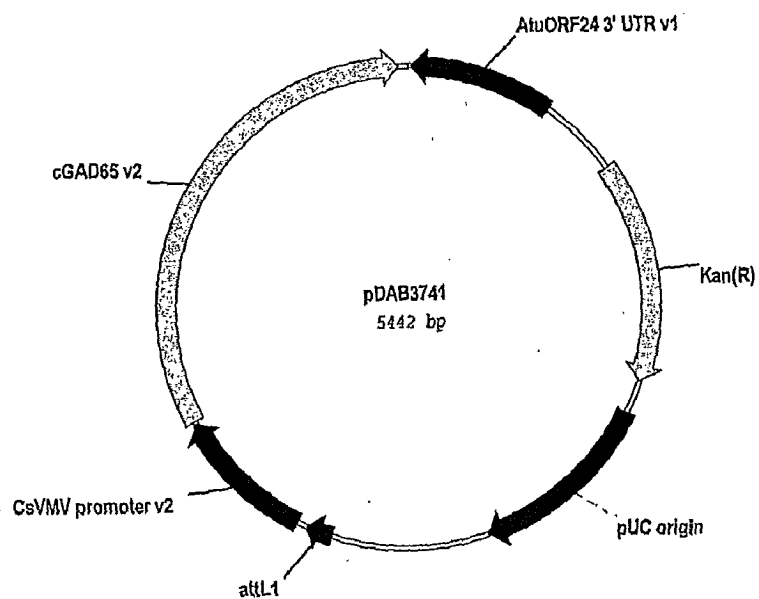


FIGURE 9H

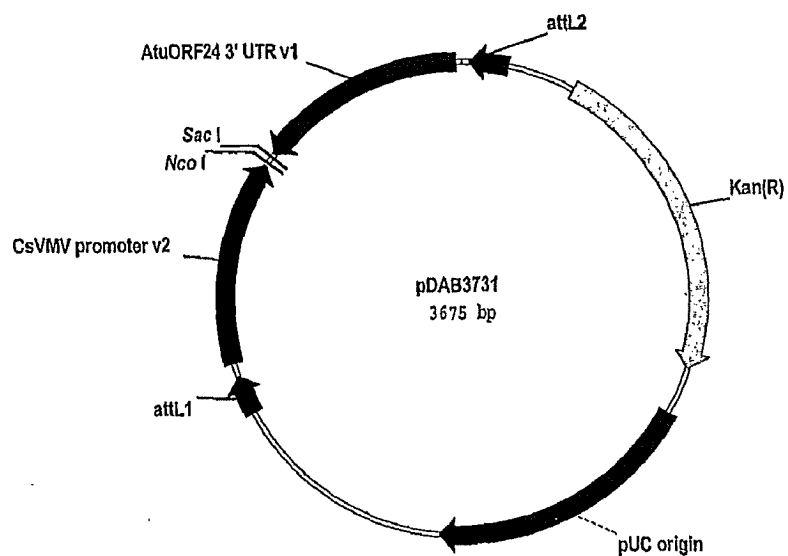


FIGURE 9I

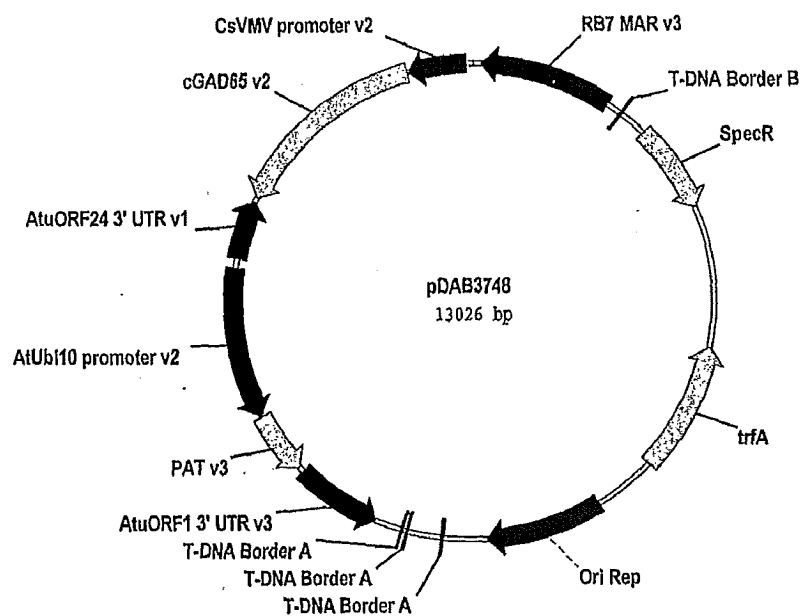


FIGURE 9J

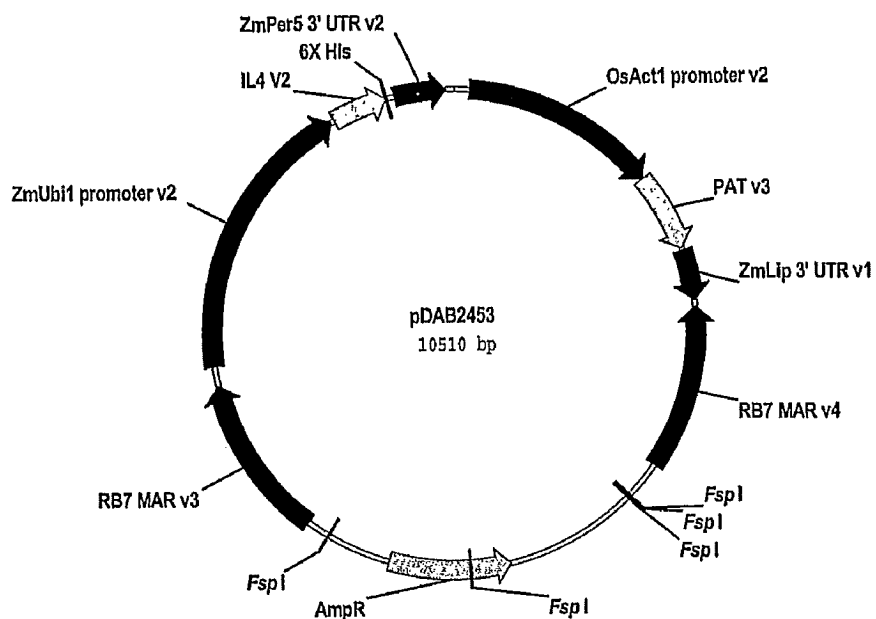


FIGURE 9K

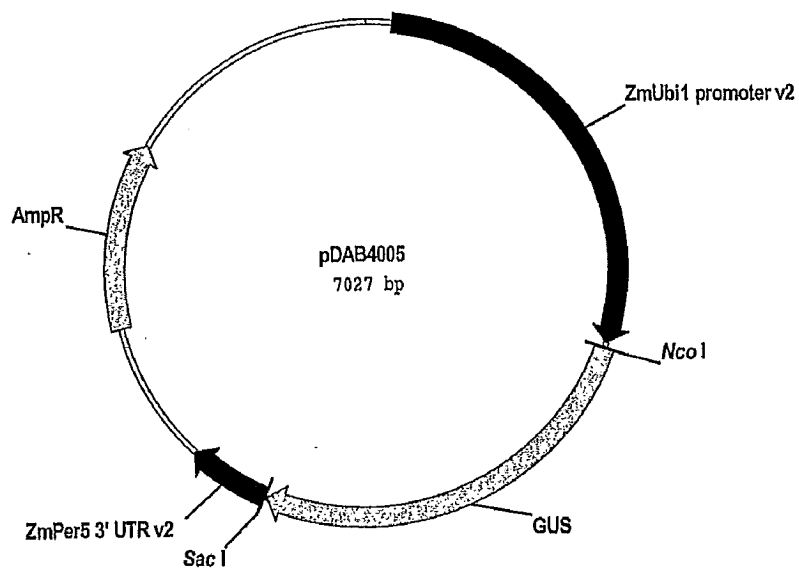


FIGURE 9L

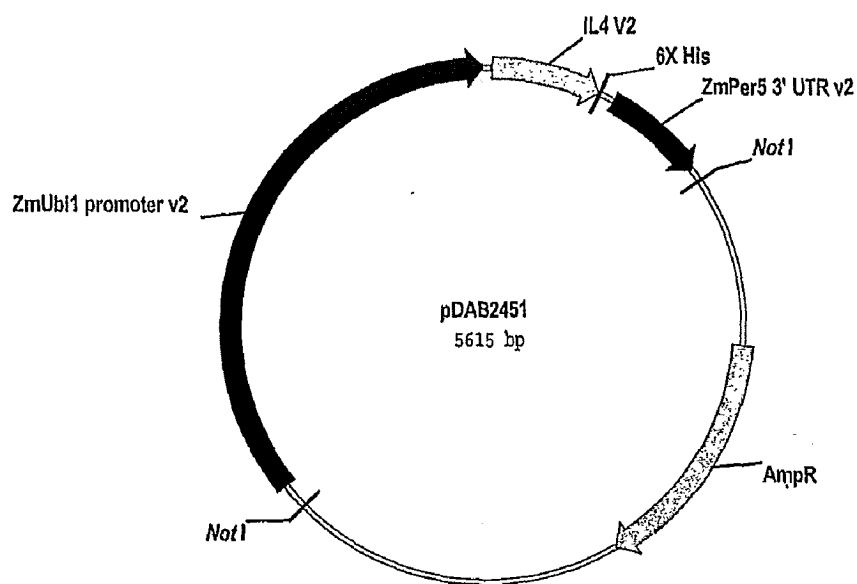


FIGURE 9M

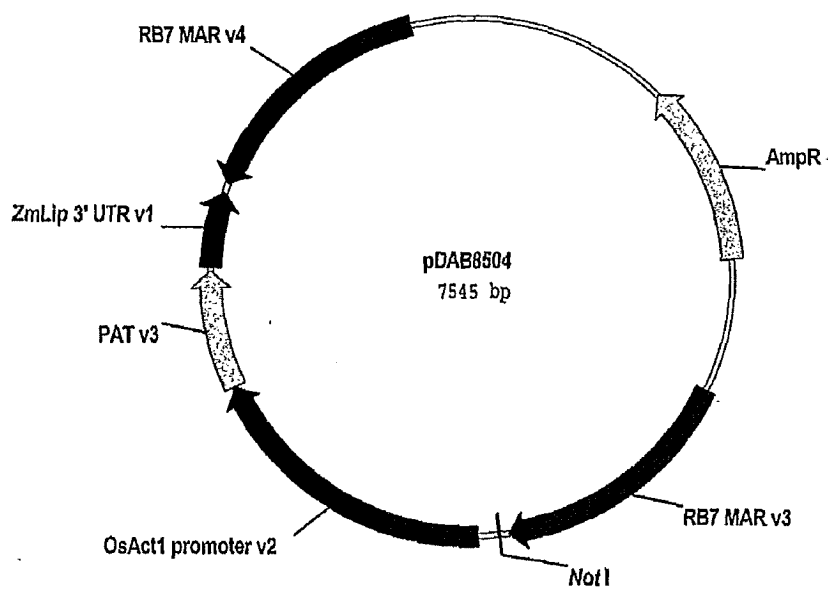


FIGURE 9N

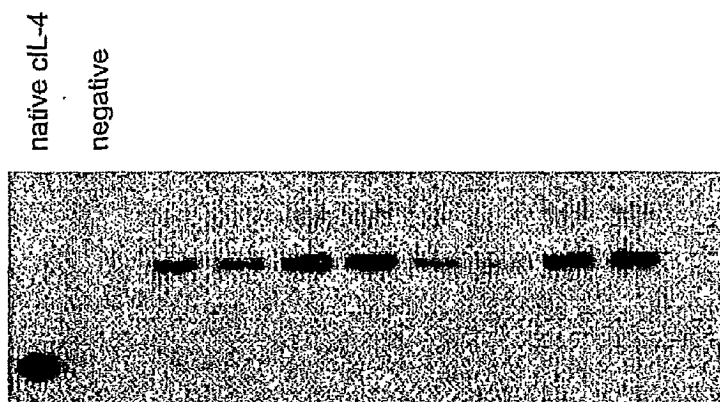


FIGURE 10

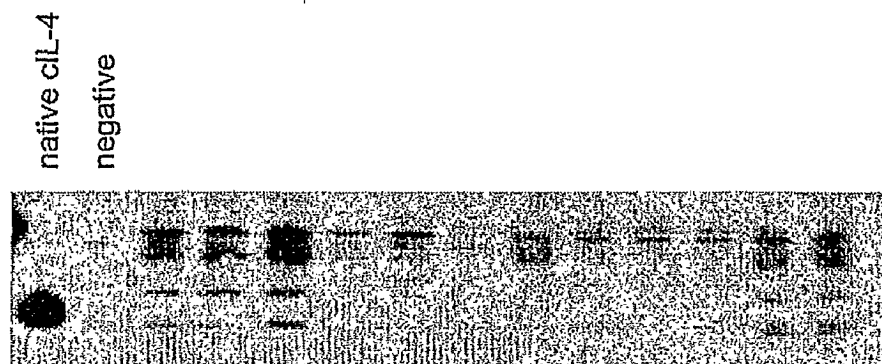


FIGURE 11

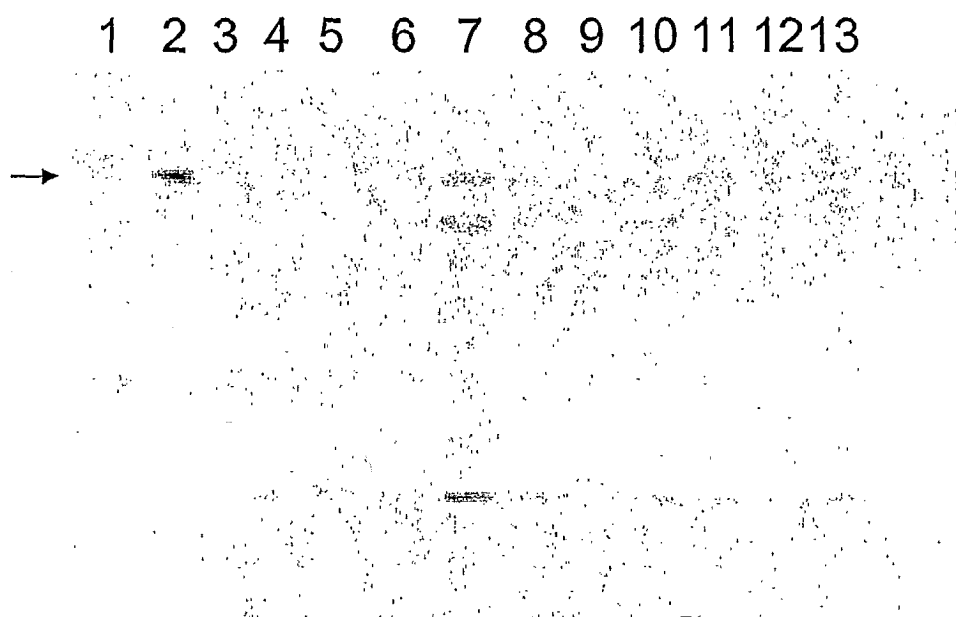


FIGURE 12

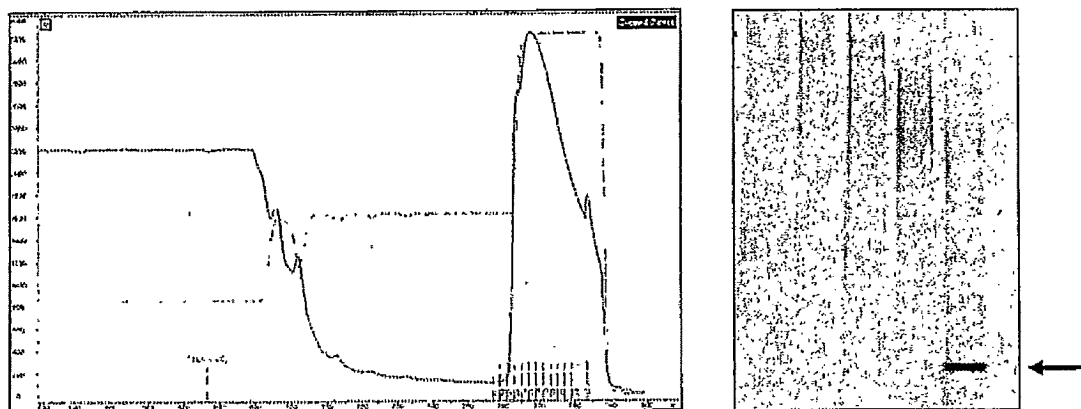


FIGURE 13

ANTI-T CELL AND AUTOANTIGEN TREATMENT OF AUTOIMMUNE DISEASE

FIELD OF THE INVENTION

[0001] The invention is directed to the treatment of autoimmune disease in mammals. More specifically the invention is directed to a new method for the treatment and diagnosis of new onset Type I diabetes in mammals.

BACKGROUND OF THE INVENTION

[0002] Type 1 or Insulin Dependent Diabetes Mellitus (IDDM) is an autoimmune disorder mainly of glucose metabolism. Complications of diabetes impair the longevity and quality of life, and include atherosclerotic heart disease, gangrene and stroke, as well as diabetic retinopathy, neuropathy and nephropathy. Symptoms of diabetic neuropathy range from peripheral sensory deficits (pins and needles/carpal tunnel syndrome) to autonomic neuropathy resulting in bladder and bowel dysfunction. Type 1 diabetes is also responsible for a large proportion of the patients on renal dialysis, the result of diabetes-induced end stage renal disease. The prevalence of myocardial infarction, angina and stroke is 2-3 times greater than in non-diabetics, and the Type 1 diabetic's life span is also shortened.

[0003] Type I diabetes actually begins before the clinical manifestations of the disease. It starts with the progressive destruction of beta cells in the pancreas. These cells normally produce insulin. The reduction of insulin response to glucose can be measured during this period, however, ultimately there is massive (>90%) destruction of beta cells in the islets of Langerhans. During the early stages of the disease and beyond, Type I diabetes is characterized by the infiltration of pancreatic islets by macrophages and lymphocytes (helper and killer). The macrophage infiltration is believed to prompt the infiltration of small lymphocytes. While clinicians understand the potential for a drug that can address macrophage involvement early in the disease, no safe therapies have yet been found. Current treatment involves daily frequent injections of insulin. However, this can lead to side effects such as hypoglycemic shock. It is important in the treatment of diabetes to control the blood sugar level and maintain it at a normal level.

[0004] Diabetes mellitus is not limited to humans but is also one of the most common endocrinopathies in dogs and cats being associated with considerable morbidity and mortality. Diabetic animals are subject to many of the same problems described in human diabetics, such as increased susceptibility to infection and reduced wound healing. Furthermore, the decreased production insulin as is the case in human Type I diabetes promotes lipolysis and moderate hyperlipidemia leading to atherosclerosis. Some complications of diabetes appear to be specific for animals, in that dogs can develop rapid cataracts leading to blindness, while cats can develop an accelerated neuropathy leading to problems of leg weakness and gait disturbance.

[0005] Glycemic control in both humans and animals is critical, however control can often not be achieved except by frequent testing and administration of insulin, which is debilitating for humans and not practical in companion animals. As a result, glycemic control is impaired in diabetic animals even with insulin administration, and there is an accelerated mortality in affected animals (Bennett N. Monitoring techniques for diabetes mellitus in the dog and the cat. Clin Tech Small

Anim Pract. 2002 May; 17(2):65-9). Treatment options for animals are currently limited to daily insulin administration as well as islet transplantation which has variable success and requires daily immunosuppression which is costly and in itself has additional toxicities (Salgado D, Reusch C, Spiess B. Diabetic cataracts: different incidence between dogs and cats. Schweiz Arch Tierheilkd. 2000 June; 142:349-53). Long term treatment of diabetic dogs with bovine or porcine insulin can lead to significant reactivity and antibodies which can cross-react with homologous insulin and thus problems in diabetic management. (Davison L J, Ristic J M, Herrtage M E, Ramsey I K, Catchpole B. Anti-insulin antibodies in dogs with naturally occurring diabetes mellitus. Vet Immunol Immunopathol. 2003 Jan. 10; 91(1):53-60).

[0006] The immunopathogenesis of diabetic disease in dogs is also very similar to human Type I diabetes, with evidence that injury is mediated primarily by autoreactive lymphocytes. Histopathologic and immunocytochemical studies of pancreas of dogs with spontaneous diabetes mellitus shows extensive pancreatic damage, marked reduction or absence of insulin producing beta cells but with preservation of alpha and delta cells. Also, insulinitis lesions are composed of infiltrating mononuclear cells, predominantly lymphocytes but evidence of islet-directed humoral autoimmunity is not detected. (Alejandro R, Feldman E C, Shienvold F L, Mintz D H. Advances in canine diabetes mellitus research: etiopathology and results of islet transplantation. J Am Vet Med Assoc. 1988 Nov. 1; 193:1050-5). T cell responses appear to be directed to autoantigens such as GAD, insulin, and IA-2, again similar to human disease. Considerable speculation exists as the potential for molecular mimicry to have precipitated autoimmune attack to islet beta cells, with exposure to viral infections. T-cell activation by rotavirus and possibly other viruses, and dietary proteins, could trigger or exacerbate beta-cell autoimmunity through molecular mimicry with IA-2 and for rotavirus—GAD. (Honeyman M C, Stone N L, Harrison L C. T-cell epitopes in type 1 diabetes autoantigen tyrosine phosphatase IA-2: potential for mimicry with rotavirus and other environmental agents. Mol Med. 1998 4:231-9). Susceptible animals may be identified by antibody screening for various diabetes autoantigens such as GAD, IA-2 and insulin.

[0007] Various therapies have been developed to try to reverse Type I diabetes. Anti-CD3 monoclonal antibodies (mAb) have been utilized to try to suppress immune responses by transient T-cell depletion and antigenic modulation of the CD3/T-cell receptor complex. Anti CD3 mAb applied to adult NOD females (a model of Type I diabetes) within 7 days of the onset of full-blown diabetes produced over 4 months remission of overt disease in most of the mice. The immunosuppression was specific for beta-cell-associated antigens (Chatenoud L, Thervet E, Primo J, Bach J F. Anti-CD3 antibody induces long-term remission of overt autoimmunity in non-obese diabetic mice. Proc Natl Acad Sci USA. 1994 91:123-7). However, there was progressive increase in the incidence of diabetes in treated mice to 4 months and full analysis beyond this time was not shown. There was as well return of insulinitis within several weeks of treatment and thus it appears that protection with anti-CD3 antibody alone was not sufficient for disease treatment or reversal. Similarly, in human studies treatment with a non-activating anti CD3 mAb maintained or improved insulin production after one year in 9 of the 12 patients in the treatment group. However, maximal benefit as evidenced by

reduced insulin requirements and lower glycated hemoglobin levels was observed at 6 months as compared to 12 months. As well only 2 of the 12 patients had a sustained response ($P=0.01$) beyond 1 year demonstrating that additional therapy would be required (Herold K C, Hagopian W, Auger J A, Pournian-Ruiz E, Taylor L, Donaldson D, Gitelman S E, Harlan D M, Xu D, Zivin R A, Bluestone J A. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. *N Engl J. Med.* 2002 May 30; 346:1692-8).

[0008] Oral immune tolerance is a process by which oral administration of protein antigens can result in diminished peripheral immune responses to a subsequent systemic challenge with the same antigen. The basis for such a regulatory system in mammals is to balance protective mucosal antibody responses to pathogens and attenuating potentially harmful allergic responses to newly encountered food proteins. Oral immune tolerance has, also been viewed as a potential therapeutic strategy for preventing and treating autoimmune diseases such as diabetes when triggering autoantigens such as glutamic acid decarboxylase (GAD) have been identified.

[0009] The use of plants as an expression system or "bioreactor" in the production of mammalian antigenic proteins for clinical use offers several unique advantages including high production capacity with near unlimited scale up. Being eukaryotes, plants can also perform post-transcriptional and post-translational modifications required for functional transgenic proteins such as formation of disulfide bonds and folding. As protein isolation costs can eliminate the economic advantage of any production system, an additional practical advantage of transgenic plants for oral tolerance is that plant expression systems can also become effective delivery systems without extensive purification. The composition of plants contains additional compounds, proteins, lectins and other moieties that participate in altering immune responses with the potential to enhance oral tolerance. As well, augmented immune responses to plant produced vaccines may suggest increased stability for plant expressed transgenic proteins to gastrointestinal degradation, and collectively these features make plants an ideal expression and delivery system for oral immune tolerance.

[0010] U.S. Pat. No. 6,338,850 discloses a method for oral immune tolerance utilizing a diabetes-associated beta cell autoantigen produced in transgenic plants. Non-obese diabetic (NOD) mice were protected from diabetes when administered such transgenic plant tissue.

[0011] In summary, although much progress has been made in the last three decades to understand the mechanisms of Type I diabetes, there is a continual need to develop new and better therapies to treat and possibly reverse the disease in both humans and animals.

SUMMARY OF THE INVENTION

[0012] The present invention is based upon the novel demonstration that the combination of anti-T cell therapy with oral immune tolerance provides a therapy that is more efficacious than either therapy used alone, for the treatment of autoimmune disease and in particular, for the treatment of Type I diabetes.

[0013] It is an aspect of the invention to provide a regimen that provides therapeutic benefit to mammals with new onset autoimmune disease such as Type I diabetes and that overcomes some of the disadvantages of currently employed therapies. It is another aspect of the invention to provide improved alternative therapies and regimens for the treatment

of Type I diabetes. These and other objectives are accomplished by the present invention which is a novel method for the treatment of new onset Type I diabetes or for the preventative treatment of those at imminent risk for developing Type I diabetes.

[0014] The method combines anti-T cell therapy with immune tolerance and is to be for administration to mammals at imminent risk (i.e. pre-diabetic) for developing Type I diabetes or those with new onset Type I diabetes. In embodiments of the invention, the method can be conducted concurrently or sequentially. As a sequential therapy the mammal is first treated with anti-T cell therapy followed by immune tolerance therapy to maintain a disease free state. The method of the invention can be used in combination with any other known diabetic treatments.

[0015] The invention is also directed to methods of diagnosis of new onset Type I diabetes in mammals. Such diagnosis comprises the detection of antibodies to for example, glutamic acid decarboxylase (GAD), as a predictor of the development of Type I diabetes. In this aspect, antibodies directed to various forms of GAD may be used in the method. In further aspects, novel gene sequences and novel antibodies directed to novel forms of GAD such as but not limited to GAD65 may be used in the invention. In still further aspects the GAD65 may be canine GAD65 and plant codon optimized genes encoding canine GAD65 as described herein.

[0016] According to an aspect of the invention is a treatment regime for Type I diabetes wherein said regime comprises the administration of anti-T cell antibodies and a composition comprising one or more autoantigens with one or more immunoregulatory cytokines to a mammal. The administration may be concurrent or sequential. The treatment regime may be used in conjunction with other known treatments for Type I diabetes. Further, the treatment regime can be used for those mammals that are at imminent risk for developing Type I diabetes.

[0017] The method of the invention comprises the use of anti-T cell therapy in conjunction with an autoantigen. However, in further aspects of the invention, the autoantigen portion of the therapy may be used alone or with a mucosal antigen such as an immunoregulatory cytokine.

[0018] In all aspects of the invention, the combined use of anti-T cell therapy and autoantigen may be concurrent or sequential. Concurrent therapy is understood by one of skill to involve the administration of anti-T cell therapy with the administration of autoantigen, alternatively, this could mean the administration of anti-T cell therapy together with the administration of autoantigen and then this may also be followed with the further administration of further autoantigen. Concurrent type of administration may be for different time periods as is understood by one of skill in the art and may be followed by further autoantigen therapy for different time periods.

[0019] According to an aspect of the present invention is a method for treating Type I diabetes in a mammal or for treating mammals at imminent risk for developing Type I diabetes, the method comprising the combined use of anti-T cell therapy with autoantigen therapy. In aspects, the use may be concurrent or sequential or a combination of both provided at different time intervals.

[0020] According to an aspect of the present invention there is a treatment regime for treating Type I diabetes in a mammal or for mammals at imminent risk for developing Type I diabetes, said method comprising:

[0021] (a) administering anti-T cell therapy to said mammal; and

[0022] (b) administering an effective immunosuppressive dose of a composition comprising at least one autoantigen;

[0023] wherein said administering of (a) and (b) is done concurrently or sequentially.

[0024] In aspects of the invention, the administration of (b) can be further continued for days and up to several days, weeks, months or years as required.

[0025] According to an aspect of the present invention there is a treatment regime for treating Type I diabetes in a mammal or for mammals at imminent risk for developing Type I diabetes, said method comprising:

[0026] (a) administering anti-T cell therapy to said mammal; and

[0027] (b) administering an effective immunosuppressive dose of a composition comprising at least one autoantigen;

[0028] wherein (a) and (b) are administered at the same time; or (a) is administered before (b); or (a) and (b) are administered at the same time and then (b) is further administered for an extended period of time.

[0029] According to an aspect of the present invention there is a treatment regime for treating Type I diabetes in a mammal or for mammals at imminent risk for developing Type I diabetes, said method comprising:

[0030] (a) administering anti-T cell therapy to said mammal; and

[0031] (b) administering an effective immunosuppressive dose of a composition comprising at least one autoantigen and at least one mucosal antigen;

[0032] wherein said administering of (a) and (b) is done concurrently or sequentially.

[0033] In aspects, (a) and (b) are administered at the same time; or (a) is administered before (b); or (a) and (b) are administered at the same time and then (b) is further administered.

[0034] According to a further aspect of the present invention there is provided a method for treating Type I diabetes in a mammal or for mammals at imminent risk for developing Type I diabetes, said method comprising: I

[0035] (a) administering an effective immunosuppressive dose of anti-T cell antibodies to said mammal; and

[0036] (b) administering an effective immunosuppressive dose of at least one autoantigen and at least one immunoregulatory cytokine;

[0037] wherein said administering of (a) and (b) is done concurrently or sequentially.

[0038] In aspects, (a) and (b) are administered at the same time; or (a) is administered before (b); or (a) and (b) are administered at the same time and then (b) is further administered.

[0039] According to an aspect of the present invention there is provided a method for treating Type I diabetes in a mammal or for mammals at imminent risk for developing Type I diabetes, said method comprising:

[0040] (a) administering an effective immunosuppressive dose of anti-T cell antibodies to said mammal; and

[0041] (b) administering an effective immunosuppressive dose of a transgenic plant material to said mammal, said transgenic plant material containing at least one autoantigen and an immunoregulatory cytokine;

[0042] wherein said administering of (a) and (b) is concurrently done.

[0043] In aspects, (b) may be further administered.

[0044] According to another aspect of the present invention there is provided a method for treating Type I diabetes in a mammal or for mammals at imminent risk for developing Type I diabetes, said method comprising:

[0045] (a) administering an effective immunosuppressive dose of anti-T cell antibodies to said mammal; and

[0046] (b) administering an effective immunosuppressive dose of a transgenic plant material to said mammal, said transgenic plant material containing at least one autoantigen and an immunoregulatory cytokine,

[0047] wherein said administering of (a) is done first and then administering of (b) is followed.

[0048] According to another aspect of the present invention there is provided a method for treating Type I diabetes in a mammal or for mammals at imminent risk for developing Type I diabetes, said method comprising:

[0049] (a) administering an effective immunosuppressive dose of anti-CD3 monoclonal antibodies to said mammal; and

[0050] (b) administering an effective immunosuppressive dose of a transgenic plant material to said mammal, said transgenic plant material containing a combination of a GAD isoform and IL-4,

[0051] wherein said administering of (a) and (b) is concurrently done.

[0052] According to another aspect of the present invention there is provided a method for treating Type I diabetes in a mammal, said method comprising:

[0053] (a) administering an effective immunosuppressive dose of anti-CD3 monoclonal antibodies to said mammal; and

[0054] (b) administering an effective immunosuppressive dose of a transgenic plant material to said mammal, said transgenic plant material containing a combination of a GAD isoform and IL-4,

[0055] wherein said administering of (a) is done first and then administering of (b) is followed.

[0056] According to another aspect of the invention is a method for the reversal of Type I diabetes in a human or animal, said method comprising:

[0057] administering a therapeutically effective amount of anti-CD3 monoclonal antibody to said human or animal; and

[0058] administering a therapeutically effective amount of a transgenic plant material containing one or more GAD autoantigens together with IL-4,

[0059] wherein said monoclonal antibody is first administered to said human or animal.

[0060] According to another aspect of the invention is a method for the reversal of Type I diabetes in a human or animal, said method comprising:

[0061] administering a therapeutically effective amount of anti-CDS monoclonal antibody to said human or animal; and

[0062] administering a therapeutically effective amount of a transgenic plant material containing one or more GAD autoantigens together with IL-4,

[0063] wherein said monoclonal antibody and said transgenic plant material is administered concurrently to said human or animal.

[0064] According to yet another aspect of the present invention is a composition comprising a mixture of anti-CD3

antibodies and a preparation that contains at least one autoantigen and an immunoregulatory cytokine.

[0065] According to yet another aspect of the present invention is a composition comprising a mixture of anti-CD3 antibodies and a transgenic plant material that contains at least one autoantigen and an immunoregulatory cytokine.

[0066] According to another aspect of the present invention is a method for the diagnosis of Type I diabetes in a mammal, the method comprising detecting in a sample from said mammal the presence of anti-GAD antibodies. Such detection being an early indicator of the development or the risk of development of Type I diabetes in the mammal. In aspects of the invention, the method may comprise the use of canine GAD65.

[0067] According to a further aspect of the present invention are novel GAD65 sequences, such sequences may be used for plant transformation. In aspects, the sequence is a canine GAD65 sequence of SEQ ID NO:4. In further aspects, the sequence is an optimized GAD65 sequence of SEQ ID NO:5.

[0068] According to still further aspects of the present invention are novel IL4 sequences, such sequences may be used for plant transformation. In aspects, the sequence is a canine IL4 sequence optimized for plant expression and is represented by SEQ ID NO:2 or SEQ ID NO:7.

[0069] In other aspects of the invention are vectors for the transformation of plant cells. In aspects, these vectors contain sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 AND SEQ ID NO. 6.

[0070] According to still a further aspect of the present invention is the use of a composition comprising anti-T cell antibodies, autoantigen and optional mucosal antigen in the manufacture of a medicament for the treatment of Type I diabetes in a mammal.

[0071] Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from said detailed description.

BRIEF DESCRIPTION OF THE FIGURES

[0072] The present invention will become more fully understood from the description given herein, and from the accompanying drawings, which are given by way of illustration only and do not limit the intended scope of the invention.

[0073] FIG. 1 shows the effect of control plant feeding versus GAD/IL-4 plant feeding on blood glucose levels in diabetic female NOD mice.

[0074] FIG. 2 shows a Kaplan Meier Survival analysis demonstrating the time to hyperglycemia for the diabetic female NOD mice of FIG. 1.

[0075] FIG. 3 shows blood glucose levels post feeding at baseline, day 40 and day 60 after anti CD3 therapy.

[0076] FIG. 4 shows the level of anti-GAD IgG1 in GAD/IL-4 fed mice compared to controls.

[0077] FIG. 5 shows the delayed mean time to diabetes for GAD/IL-4 fed mice compared to controls.

[0078] FIG. 6 shows the levels of serum anti-GAD65 antibodies in healthy and newly diagnosed diabetic dogs by anti-GAD ELISA.

[0079] FIG. 7 shows Western blot analysis of canine GAD65 protein expression in transgenic tobacco plants. Total protein extracts (40 µg/lane) from transgenic tobacco leaf tissue were fractionated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted on to polyvinylidene difluoride (PVDF) membrane and probed with an anti-GAD antibody. Lanes 1 to 5, independent canine GAD65 transgenic tobacco lines; WT, wild-type tobacco; GAD67, used as a positive control. Numbers on the left indicate the positions of protein size markers.

[0080] FIG. 8A shows that plant rcIL-4 stimulates the ³H-thymidine incorporation by TF-1 cells in a dose-dependent manner. Pre-incubation of plant rcIL-4 with anti-canine IL-4 antibody (Ab) reduces its ability to stimulate TF-1 cells in proliferation. FIG. 8B shows the incorporation of ³H-thymidine by TF-1 cells in response to stimulation with a standard commercial source of rcIL-4.

[0081] FIGS. 9A-9N are various plasmid constructs used for cell transformations. FIG. 9A pDAB771; FIG. 9B pDAB773; FIG. 9C pDAB2407; FIG. 9D pDAB2457; FIG. 9E pDAB2455; FIG. 9F pDAB2456; FIG. 9G pDAB3736; FIG. 9H pDAB3741; FIG. 9I pDAB3731; FIG. 9J pDAB3748; FIG. 9K pDAB2453; FIG. 9L pDAB4005; FIG. 9M pDAB2451; and FIG. 9N pDAB8504.

[0082] FIG. 10 shows western blots of IL-4 expression in transgenic tobacco. Calli were extracted in SDS gel loading solution and heated at 95° C. Gels and westerns were performed as described in the examples section.

[0083] FIG. 11 shows western blots of IL-4 expression in transgenic rice. Calli were extracted in SDS gel loading solution and heated at 95° C. Gels and westerns were performed as described in the examples section.

[0084] FIG. 12 shows western analysis of the cGAD65 samples expressed in NT-1 calli. Calli were extracted in SDS gel loading solution and heated at 95° C. The arrow indicates the recombinant standard rhGAD65. Lane 1: molecular weight markers; lane 2: rhGAD65 standard; lane 3: non-transgenic callus; lanes 4-13: independent cGAD65 transgenic events.

[0085] FIG. 13 shows the purification of IL-4. cIL-4 produced in transgenic tobacco callus was purified as described above. The chromatograph of the Hi-Trap Nickel column is shown, with the fractions retained for further purification. SDS-PAGE analysis of the fractions eluted from the Superose 6 column identified a major protein band (arrow) that corresponded to cIL-4 as determined by western blot and MALDI-TOF analysis.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0086] The invention is a new treatment method/regime for autoimmune disease and in particular, for the treatment of Type I diabetes in mammals. The method is a combination treatment whereby anti-T cell therapy is combined with immune tolerance in a mammal. The combination treatment may be done concurrently or sequentially. The combination of anti-T cell therapy with immune tolerance provides a therapy that is more efficacious than either therapy alone, particularly for the treatment of Type I diabetes. Using the method of the present invention, it was demonstrated that there was no reverting to diabetes for an extensive period of time demonstrating that the method is an effective long term treatment and can in fact reverse diabetes.

[0087] The method of the invention is useful for the treatment of new onset Type I diabetes in a mammal. The method of the invention is also useful for the treatment of mammals at imminent risk for developing Type I diabetes which includes mammals with impaired islet cell function due to autoimmunity but not requiring insulin therapy. In this aspect, such mammals are considered pre-diabetic. The method can be used to reverse Type I diabetes in mammals.

[0088] It is understood by those of skill in the art that the method of the invention can be used in conjunction with other known treatments suitable for Type I diabetes.

DEFINITIONS

[0089] As referred to herein, Type 1 diabetes is generally understood to be an auto-immune disease and is also referred to in the general literature as: type 1 DM, insulin-dependent diabetes, IDD, insulin-dependent diabetes mellitus, IDDM, childhood diabetes, childhood diabetes mellitus, childhood-onset diabetes, childhood-onset diabetes mellitus, diabetes in childhood, diabetes mellitus in childhood, juvenile-onset diabetes, juvenile-onset diabetes mellitus, and autoimmune diabetes mellitus.

[0090] As referred to herein, autoantigens are native proteins or peptides that, in some individuals, are immune response-provoking. When autoantigens are administered to such individuals, the autoantigens induce tolerance or suppress the immune response of the mammal to the protein or peptide.

[0091] As referred to herein, mucosal adjuvants are immunological agents which work through or at the mucosal surface, or at lymphoid structures associated with the gut and increase the antigenic response. Mucosal adjuvants as disclosed herein may be immunoregulatory cytokines that are any of several regulatory proteins, such as cytokines and interleukins that are released by cells of the immune system and act as intercellular mediators in the generation of an immune response. Cytokines may include those released by lymphocytes, other immune cells or parenchymal cells upon activation which can modify, attenuate or eliminate harmful autoimmune responses directed to a specific antigen or antigens.

[0092] As referred to herein, GAD (glutamic acid decarboxylase) encompasses different GAD isoforms as well as GAD polypeptides that contain one or more GAD epitopes recognized by autoantibodies.

[0093] As referred to herein, the term "transgenic plant material" as used herein is any type of transgenic plant material that contains the autoantigen and mucosal adjuvant as expressed by the plant. The plant material may include but not be limited to plant tissue, plant part (i.e. leaves, tubers, stems etc.), plant cell cultures including but not limited to plant suspension cultures and plant callus cultures, plant extracts, plant slurries and combinations thereof. The plant material can be provided "raw" or processed in some manner so long as it contains the transgenic protein of interest. Methods for processing plant material that are consistent with use in the present invention may be found in WO2002083072, WO2004098530, and WO2004098533 (the disclosures of which are herein incorporated by reference in their entirety).

[0094] As referred to herein, "mammal" includes any warm-blooded animal with mammary glands. A preferred group of mammals is the group consisting of humans and companion animals. In aspects, this group consists of

humans, dogs, cats and horses. In preferred aspects, this group consists of dogs and cats and in most preferred aspects this group is humans.

Anti-T Cell Therapy

[0095] In the method, the anti-T cell therapy is administered to the mammal to cause T cell depletion. The anti-T cell therapy may be any effective immunosuppressant agent that targets T cells. In aspects of the invention this may include but not be limited to monoclonal antibodies and polyclonal antibodies that target surface antigens on T cells or alternatively other agents such as cyclosporine, methotrexate and azathioprine.

[0096] In aspects of the invention suitable antibodies may be selected from but not be limited to anti CD3, anti CD2, anti CD4, anti CD7, anti CD8, anti CD25, anti CD28, alpha 4 beta 1 integrin, alpha 4 beta 7 integrin and other T cell surface antigens as is well understood by those of skill in the art. The selected T cell depletion agent such as an antibody as herein described would then be administered to the mammal in need thereof. Treatment with the antibody would be done for up to about 10 days. This time period can be varied as is understood by one of skill in the art. In aspects, this time period can be about 5 to 7 days.

[0097] The T cell depletion agent may be administered at dosages of about 10 µg/kg to about 100 µg/kg body weight intravenously. This may include any range therebetween such as but not limited to 10 µg/kg to about 20 µg/kg body weight; 20 µg/kg to about 30 µg/kg body weight; 30 µg/kg to about 40 µg/kg body weight; 40 µg/kg to about 50 µg/kg body weight; 50 µg/kg to about 60 µg/kg body weight; 60 µg/kg to about 70 µg/kg body weight; 70 µg/kg to about 80 µg/kg body weight; 80 µg/kg to about 90 µg/kg body weight; and 90 µg/kg to about 100 µg/kg body weight. It is also understood by one of skill in the art that the dose range may differ from the described range and thus may not be limited to this range depending on the species of mammal and should be a dosage that essentially eliminates circulating T cells, as measured in peripheral blood. Determination of the suitable dose of T cell depletion agent may be accomplished by detection of monoclonal antibodies on the surface of circulating T cells. Responsiveness to the antibody treatment may be confirmed by measurement of blood sugar levels whereby mammals exhibiting levels under control and within normal ranges are then considered to be responsive to the treatment.

[0098] In one representative but non-limiting embodiment of the invention, anti-T cell therapy is effectively accomplished by the administration of anti-CD3 monoclonal antibodies for about 5 to about 7 days.

Immune Tolerance

[0099] The method of the invention also incorporates immune tolerance. Immune tolerance is achieved by administering to the mammal one or more autoantigens and, optionally, one or more mucosal adjuvants. The autoantigen and optional mucosal adjuvant may be co-administered when a mucosal adjuvant is used. Immune tolerance can be administered concomitantly with the anti-T cell therapy or after the anti-T cell therapy is completed. Furthermore, immune tolerance treatment may be administered after concurrent first administration of the combined anti-T cell therapy and oral tolerance. In other words, the autoantigen and optional mucosal antigen can be administered for a time period as required. Thus the autoantigen may be administered for an extended period of time to the mammal in need of the treatment that is well beyond the time of anti-T cell therapy

administration. In some aspects this may be the lifespan of the mammal if continued administration is required.

[0100] If administered after completion of the anti-T cell therapy, oral immune tolerance can be delayed for up to about 4 weeks. In aspects of the invention, the immune tolerance is mucosal immune tolerance whereby the autoantigen and mucosal adjuvant are co-administered via a mucosal surface. In these aspects of the invention a preferred mucosal immune tolerance is induced orally.

[0101] The autoantigen selected is the trigger antigen responsive for the autoimmune disease. In the case of Type I diabetes, the autoantigen is selected from the group consisting of species specific or species non-specific GAD (glutamic acid decarboxylase) isoforms and GAD polypeptides. GAD isoforms are known to those of skill in the art and may include but not be limited to GAD65 and GAD67. Still other autoantigens may be selected from the group consisting of insulin and beta cell proteins capable of eliciting harmful autoimmune responses. The amount of autoantigen that may be used for administration was found to be about 7-8 $\mu\text{g}/25$ gm mouse. Thus the amount of autoantigen for use in the method of the invention is about up to 300 $\mu\text{g}/\text{kg}$ for a mammal and any ranges therebetween. Thus suitable amounts may include but not be limited to about 1 $\mu\text{g}/\text{kg}$ to 1000 $\mu\text{g}/\text{kg}$; 10 $\mu\text{g}/\text{kg}$ to 800 $\mu\text{g}/\text{kg}$; about 50 $\mu\text{g}/\text{kg}$ to 700 $\mu\text{g}/\text{kg}$; about 100 $\mu\text{g}/\text{kg}$ to 500 $\mu\text{g}/\text{kg}$; and about 200 $\mu\text{g}/\text{kg}$ to 400 $\mu\text{g}/\text{kg}$. Dosage amounts for a particular mammal may be varied as is understood by one of skill in the art.

[0102] It is also understood by those of skill in the art that the GAD sequences used in the present invention may be of any species such as but not limited to human, feline and canine sequences. The human sequence is disclosed in Bu et al., 1992. Two human glutamate decarboxylases, 65-kDa GAD and 67-kDa GAD are each encoded by a single gene. Proc Natl Acad Sci USA 89:2115-2119 (the disclosure of which is incorporated herein by reference in its entirety). The feline GAD sequence is disclosed in Kobayashi et al., 1987. Glutamic acid decarboxylase cDNA: nucleotide sequence encoding an enzymatically active fusion protein. J. Neurosci. 7:2768-2772 (the disclosure of which is incorporated herein by reference in its entirety). Canine sequences for use in the invention may include those of native canine GAD65 (SEQ ID NO.4) and canine GAD65 (SEQ ID NO.5) having a poly-histidine purification tag which was codon optimized for plant expression.

[0103] The GAD peptide sequences for use in the invention may be obtained by chemical synthesis using automated instruments or alternatively by expression from nucleic acid sequences which are capable of directing synthesis of the peptide using recombinant DNA techniques well known to one skilled in the art. GAD peptides of the invention may be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, J. Am. Chem. Assoc. 85:2149-2154 (1964)) or synthesis in homogenous solution (Houbenweyl, Methods of Organic Chemistry (1987), (Ed. E. Wansch) Vol. 15, pts. I and II, Thieme, Stuttgart). Techniques for production of proteins by recombinant expression are well known to those in the art and are described, for example, in Sambrook et al. (1989) or latest edition thereof.

[0104] Also encompassed by the canine GAD nucleic acid sequences of the invention are complementary as well as anti-complementary sequences to a sequence encoding and equivalent sequence variants thereof. One skilled in the art would readily be able to determine such complementary or anti-complementary nucleic acid sequences. Also as part of the invention are nucleic acid sequences which hybridize to

one of the aforementioned nucleic acid molecules under stringent conditions. "Stringent conditions" as used herein refers to parameters with which the art is familiar and such parameters are discussed, for example, in the latest editions of Molecular Cloning: A Laboratory Manual, J. Sambrook, et al., eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., or Current Protocols in Molecular Biology, F. M. Ausubel, et al., eds., John Wiley & Sons Inc., New York. One skilled in the art would be able to identify homologues of nucleic acids encoding the BCSP peptides of the invention. Cells and libraries are screened for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

[0105] It is noted that the nucleic acid molecules described herein may also encompass degenerate nucleic acids. Due to degeneracy in the genetic code, variations in the DNA sequence will result in translation of identical peptides. It is thus understood that numerous choices of nucleotides may be made that will lead to a sequence capable of directing production of the peptides or functional analogues thereof of the present invention. As a result, degenerative nucleotide substitutions are included in the scope of the invention.

[0106] Allowing for the degeneracy of the genetic code as well as conserved and semi-conserved substitutions, sequences which have between about 40% and about 80%; or more preferably, between about 80% and about 90%; or even more preferably, between about 90% and about 99%; of nucleotides which are identical to the nucleotides of SEQ ID NO:2, 4, 5 and 7 will be sequences which are "essentially as set forth in SEQ ID NO:2, 4, 5 and 7". Sequences which are essentially the same as those set forth in SEQ ID NO:2-4, 5 and 7 may also be functionally defined as sequences which are capable of hybridizing to a nucleic acid segment containing the complement of SEQ ID NO:2, 4, 5 and 7 under standard or less stringent hybridizing conditions. Suitable standard hybridization conditions will be well known to those of skill in the art.

[0107] As would be understood by one of skill in the art, nucleic acid molecules of the present invention may encompass single and double stranded forms, plasmid(s), viral nucleic acid(s), plasmid(s) bacterial DNA, naked/free DNA and RNA. A viral nucleic acid comprising a nucleic acid sequence encoding for at least one peptide of the invention may be referred to as a viral vector.

[0108] The invention also encompasses expression vectors comprising the nucleic acid sequences of the invention of SEQ ID NO. 2, 4, 5 and 7 and functional analogues thereof within expression vectors. Any expression vector that is capable of carrying and expressing the nucleic acid sequences encoding for the peptides of the invention and functional analogues thereof in prokaryotic or eukaryotic host cells may be used, including recombinant viruses such as poxvirus, adenovirus, alphavirus and lentivirus. The invention also encompasses host cells transformed, transfected or infected with such vectors to express the peptides or functional analogues of the invention. As such, host cells encompass any potential cell into which a nucleic acid of the present invention may be introduced and/or transfected.

[0109] The optional mucosal adjuvant for use in conjunction with the autoantigen may be selected from an immunoregulatory cytokine such as but not limited to the interleukins: IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15 and IL-18. Any cytokine that is released by lymphocytes, other immune cells or parenchymal cells upon activation which can modify, attenuate or eliminate harmful autoimmune responses directed to a specific antigen or antigens is suitable for use in the present invention as is

understood by one of skill in the art. In aspects of the invention, the cytokine used is IL-4 of which may be of various species origin such as for example but not limited to human (as described in Yokota et al., 1986. Proc Natl Acad. Sci USA. 83:5894-5898, the disclosure of which is incorporated by reference herein in its entirety) and canine (as described in Lee et al., 1986. Proc Natl Acad Sci USA. 83:2061-2065, the disclosure of which is incorporated herein by reference in its entirety). In these aspects a suitable canine IL-4 sequence is represented by SEQ ID NO.2 and SEQ ID NO. 7 which are optimized for plant expression. As with GAD described above, IL-4 sequences as disclosed herein may encompass various forms and be incorporated into various constructs for use in cell transfection. Suitable amounts of cytokine for use in the invention has been demonstrated to be about 1-2 $\mu\text{g}/25$ gm in the mouse. Thus in mammals suitable amounts for use in the methods are up to about 500 $\mu\text{g}/\text{kg}$ and any range therebetween such as for example about 0.5 $\mu\text{g}/\text{kg}$ to about 500 $\mu\text{g}/\text{kg}$; about 1.0 $\mu\text{g}/\text{kg}$ to about 250 $\mu\text{g}/\text{kg}$; and about 10.0 $\mu\text{g}/\text{kg}$ to about 100 $\mu\text{g}/\text{kg}$. One of skill in the art would clearly understand amounts of suitable dosages for use in the present invention.

[0110] The autoantigen and optional mucosal adjuvant may be administered as a composition. The compositions may be administered alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to the subject alone, or in combination with other agents or drugs.

[0111] The pharmaceutical compositions encompassed by the invention may be administered by any number of routes. Pharmaceutical compositions for oral and mucosal administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient. Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethylcellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

[0112] Pharmaceutical preparations which can be used orally include capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Such capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

[0113] Pharmaceutical formulations suitable for parenteral (intravenous and intramuscular) administration may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the vis-

cosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers may also be used for delivery. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[0114] The pharmaceutical compositions of the present invention may be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms.

[0115] The pharmaceutical composition may be provided in biodegradable microspheres as is disclosed in Sinha et al., Journal of Controlled Release 90 (2003) 261-280 (the disclosure of which is incorporated herein by reference).

[0116] In one embodiment of the invention, immune tolerance is achieved via a method of oral immune tolerance where the autoantigen and the mucosal adjuvant are administered within an edible plant material and as such are produced by a transgenic plant that contains the required sequences that are expressed by the plant to produce the proteins in the plant. The expression of GAD autoantigens in plants is described in U.S. Pat. No. 6,338,850 (the disclosure of which is incorporated herein by reference in its entirety). Autoantigens and mucosal adjuvants can be successfully produced in transgenic plants as is disclosed in Ma S, Huang Y, Yin Z, Menassa R, Brandle J E, Jevnikar A M Induction of oral tolerance to prevent diabetes with transgenic plants requires-glutamic acid decarboxylase (GAD) and IL-4. Proc Natl Acad Sci USA. 2004 Apr. 13; 101(15):5680-5 and in Ma S W, Zhao D L, Yin Z Q, Mukherjee R, Singh B, Qin H Y, Stiller C R, Jevnikar A M. Transgenic plants expressing autoantigens fed to mice to induce oral immune tolerance. Nat Med. 1997 July; 3(7):793-6 (the disclosures of which are incorporated herein by reference in their entirety).

[0117] Briefly, to construct a transgenic plant expressing an autoantigen, a cDNA coding for a selected autoantigen such as for example human GAD may be inserted into an expression vector and used to create transgenic plants by means of *Agrobacterium*-mediated transfection, as described herein in a representative but non-limiting example (Example A). In this example, a potato plant is used as transgenic starch tubers provide a very inexpensive source of biomass for heterologous protein production. Transgenic plants expressing the desired antigen may be identified by examination of plant extracts by Western blotting, by conventional techniques, expressed antigen being detected by means of an appropriate specific antibody. Where the antigen to which tolerance is desired has a heterodimeric structure, one may either transform plant tissue sequentially with two vectors, each carrying the DNA for an individual protein chain and a different selection in marker gene, so that the plant produces the mature antigen, or one may introduce the DNA for each chain into

separate plants and breed these, by cross-pollination of "single chain" plants by standard techniques to give hybrids producing the mature antigen.

[0118] The transgenic plant material containing the expressed antigen may be administered orally or enterally to the subject in an effective dose. The particular selection of plant for transgenic manipulation may be edible or non-edible. If a non-edible plant species is used for production of mammalian antigens, the antigens may be extracted from the plant tissue and purified as required by standard methods before oral or enteral administration.

[0119] The transgenic plant material can be administered to the mammal in need of as required. In order to produce oral tolerance in a subject to a particular mammalian antigen, transgenic plant tissue containing the expressed antigen may be administered orally or enterally to the subject in an effective dose as described herein supra. Alternatively, if a non-edible transgenic plant is used for production of mammalian antigens, the antigens may be extracted from the plant tissue and purified as required by standard methods before oral or enteral administration. This can include a single administration, multiple administration over time or continued lifetime use. Representative suitable plants for use in the invention may include but are not limited to potato, tomato, alfalfa, canola, rice, tobacco, maize, algae, safflower, moss and bryophyte.

[0120] The amount of expressed autoantigen and mucosal adjuvant when used in combination for administration to provide a therapeutic effect is provided on a weight basis and may range in combination from up to about 1 mg/kg to up to about 1000 mg/kg or more along with the plant matrix. In aspects the amount is from up to about 1 mg/kg to up to about 100 mg/kg. It is understood by those of skill in the art that the amount of expressed autoantigen and mucosal adjuvant may vary and may be selected from any sub-range of the 1 mg/kg to about 1000 mg/kg range, such as for example but not limited to; 1 mg/kg-500 mg/kg; 1 mg/kg-250 mg/kg; 1 mg/kg-200 mg/kg; 1 mg/kg-150 mg/kg; 1 mg/kg-75 mg/kg; 1 mg/kg-50 mg/kg; and 1 mg/kg-25 mg/kg and any sub-ranges of any of these ranges. Again, it is also possible that the amount may be greater than 1000 mg/kg and in some aspects less than 1 mg/kg. The amount used in the invention may be species specific as is understood by one of skill in the art.

[0121] Various methods are available to identify autoantigen and cytokine production in plants such as with the use of cross reactive monoclonal human and other species antibodies which can be applied to flow cytometric, Western blot analyses and ELISA studies (Pedersen L G, Castelruiz Y, Jacobsen S, Aasted B. Identification of monoclonal antibodies that cross-react with cytokines from different animal species. *Vet Immunol Immunopathol.* 2002 88:111-22).

[0122] The invention also encompasses therapeutic compositions comprising a mixture of T-cell immunosuppressant agent, at least one autoantigen and optionally at least one mucosal adjuvant. In a non-limiting aspect of the invention this may be represented by a composition comprising a GAD isoform, anti-CD3 monoclonal antibody and IL-4. In a further non-limiting aspect of the invention this may be represented by a composition comprising GAD65 and/or GAD67, anti-CD3 monoclonal antibody and IL-4 and/or IL-10. Such a composition may be formulated as herein described for oral or parenteral administration.

[0123] It is also understood by one of skill in the art that the method of the invention in its various embodiments can be

practiced in conjunction with other treatments currently known and used for the treatment of Type I diabetes. Such treatments may include but not be limited to insulin therapy.

[0124] The invention also encompasses the use of anti-GAD65 antibodies for the early detection of Type I diabetes. In this aspect, mammalian sera may be assayed for the presence of anti-GAD65 antibodies which is a predictor of diabetic risk or a diagnostic of Type I diabetes in early stages. In further aspects, such methods may be used in non diabetic identified animals for early detection of diabetes assessment of risk. In these aspects, various types of anti-GAD65 antibodies may be used including novel canine antibodies.

[0125] The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

EXAMPLES

Example A

The Effect of Control Plant Feeding Versus GAD/IL-4 Plant Feeding on Blood Glucose Levels in Diabetic Female NOD Mice

[0126] Diabetic female NOD mice were identified by positive urine glucose followed by blood testing. Mice were selected for antibody therapy if blood glucose was greater than 16 mmol/l on 2 consecutive days. Mice were treated with daily insulin to keep blood glucose levels less than approx. 14 mmol/l. Some mice required twice daily insulin. Mice were stabilized and given anti CD3 mAb (5 µg) IV by tail vein injection for 6 days (d1-6). Mice that failed to achieve (on 2 consecutive days), blood sugars less than 20 mmol/l by 3 weeks were terminated. More than 85% of mice achieved this. At 2 to 3 weeks following anti CD3 mAb treatment, mice were given either control plant chow (empty vector, LN tobacco) or GAD-IL4 plant chow and followed daily for blood sugars. Insulin was stopped by 3 weeks in all mice. 10 mice were assigned to each treatment group, and expressed values are mmol/l glucose vs day of experiment (FIG. 1). Day 1 indicates the start of anti CD3 therapy. One GAD/IL4 mouse had late transient hyperglycemia. One GAD/IL-4 mouse was lost to technical problem (insulin OD with hypoglycemia).

[0127] Kaplan Meier Survival Analysis Demonstrating the Time to Hyperglycemia for the Diabetic Female NOD Mice.

[0128] Diabetic female NOD mice of FIG. 1 were followed by blood testing and were given either control plant chow (empty vector, LN tobacco) or GAD-IL4 plant chow. One GAD/IL-4 mouse was lost to technical problem (insulin OD with hypoglycemia) and was not considered a treatment failure (censored). Survival was defined as time to hyperglycemia (glucose > 12 mmol/l on two consecutive days), at 40 days or beyond: 40 days was selected as mice had stabilized early fluctuations in blood glucose levels (FIG. 1) noted in most mice. The analysis is shown in FIG. 2.

[0129] Blood Glucose Levels Post Feeding

[0130] Mice were analyzed for blood glucose levels at baseline, day 40 and day 60 so that direct comparisons could

be made for a specific interval after anti CD3 therapy (FIG. 3). Day 40 levels are different ($p=0.03$) but differences also noted on day 60 did not reach significance ($p=0.1$). Note that mice at baseline were treated with insulin but at start of treatment with anti CD3 antibody. There is no difference between control and GAD-IL4 regardless at baseline. Days were selected at day 40 and 60 as there sufficient mice to assess both groups ($n=10$ at baseline and day 40, $n=6-7$ at day 60).

[0131] Production of Transgenic Plants Expressing Autoantigen

[0132] Human GAD Expression in Potatoes: Two cDNA clones encoding portions of human GAD were used, representing either the 5' sequence or the 3' sequence. The two clones had an overlap sequence of no more than 70 nt. The complete human GAD sequence was made by a series of DNA manipulations. The N-terminal end of human GAD was modified by PCR to include the incorporation of a NcoI (CCATGG) restriction site as part of a translation initiation site. The native 3' nontranslated sequence, including poly A tail, was completely removed. The modified human GAD sequence was cloned into plasmid vector pTRL-GUS to replace the GUS gene. The plasmid pTRL GU3 is composed of CaMV 35S promoter with double enhancer sequence (Ehn-35S) linked to 5' untranslated TEV leader sequence, GUS gene and NOS-terminator. The new expression cassette, consisting of 5'-Ehn35S-TEV5' untranslated leader human GAD-NOS terminator was excised with HindIII and inserted into the binary vector pBIN19. The final construct, designated pSM215, was transferred into agrobacterium and potato transformation was carried out by the leaf disc method (Horsch et al., (1985), Science, vol. 227, pp. 1229-1231). Regeneration of transformed leaf disk into new plants was according to Horsch et al. Primary screening of transformants was based on callus formation on MSO media supplemented with kanamycin.

[0133] A more complete experimental protocol for autoantigen expression in plants is provided in Ma S, Huang Y, Yin Z, Menassa R, Brandle J E, Jevnikar A M Induction of oral tolerance to prevent diabetes with transgenic plants requires glutamic acid decarboxylase (GAD) and IL-4. Proc Natl Acad Sci USA. 2004 Apr. 13; 101(15):5680-5 and in Ma S W, Zhao D L, Yin Z Q, Mukherjee R, Singh B, Qin H Y, Stiller C R, Jevnikar A M (the disclosures of which are incorporated herein by reference in their entirety). One of skill in the art could follow these teachings to practice the present invention.

Example B

Sequential Anti-CD3 and GAD/IL-4 Feeding in the NOD Mouse Model Showing Effect of Anti-CD3 and Sequential GAD/IL-4 Feeding on Th1/Th2 T Cell Subsets

[0134] Female NOD mice were allowed to spontaneously develop diabetes and maintained glycemic control with insulin. Mice were treated daily with anti-CD3 mAb (5 μ g) for 6 days and insulin was discontinued on reaching euglycemia. Mice were fed with oral GAD/IL-4, plant control diet or regular mouse chow for 4 weeks ($n=3$ per group). Mice were euthanized and serum was tested for anti-GAD IgG1 as measure of Th2 activity. GAD/IL-4 mice had the highest level of anti-GAD IgG1 antibodies ($p=0.1$) suggesting the benefit of anti-CD3 mAb with sequential GAD/IL-4 is related to Th2 skewing of T helper cell subsets.

Means Table for anti GAD IgG1 (1:1)
Effect: group

	Count	Mean	Std. Dev.	Std. Err.
plant	3	1.131	.273	.157
GAD/IL4	3	1.587	.395	.228
regular	3	1.336	.201	.116

Effect of Anti-CD3 and Sequential GAD/IL-4 on Ability to Prevent Adoptive Transfer of Diabetes

[0135] Female NOD mice were allowed to spontaneously develop diabetes and maintained glycemic control with insulin. Mice were treated daily with anti-CD3 mAb (5 μ g) for 6 days and insulin was discontinued on reaching euglycemia. Mice were fed with oral GAD/IL-4, plant control diet or regular mouse chow for 4 weeks ($n=3$ per group) and are the same as mice presented in FIG. 1. Mice were euthanized and spleen cells were isolated from mice from each group were mixed with diabetogenic spleen cells taken from recently diabetic NOD mice. Cells from fed mice (1×10^7) were mixed with diabetogenic spleen cells (1×10^7) and were injected IV into NOD-scid mice. Recipient mice were followed for diabetes by urine testing and confirmed by serum testing (>14 mmol/l for 2 days) and then terminated. Mice receiving oral GAD/IL-4 had delayed me and time to diabetes ($p=0.1$) compared to plant controls suggesting the benefit of anti-CD3 mAb with sequential GAD/IL-4 is related to induction of regulatory Th2 helper cell subsets (FIG. 5).

Means Table for day of diabetes
Effect: group

	Count	Mean	Std. Dev.	Std. Err.
plant	7	25.000	4.320	1.633
GAD/IL4	6	32.500	10.691	4.365
regular	6	27.500	7.369	3.008

The Relevance of Elevated GAD65 Antibodies to Diabetes of Non Human or Mouse Mammals.

[0136] Dogs with recent onset of insulin dependent autoimmune diabetes were tested for the presence of serum anti-GAD65 (total) antibodies using ELISA. As demonstrated, the presence of anti-GAD antibodies was highly related to diabetes as no antibodies were detected in non-diabetic normal dogs (FIG. 6). These data suggest the relevance of GAD65 in canine diabetes, and that early detection of anti-GAD65 antibodies in non diabetic dogs might be used to predict diabetic risk as well as monitoring skewing of TH2 subsets.

Expression of Canine GAD65 in Transgenic Tobacco Plants.

[0137] A plant expression vector, containing the entire coding sequence of canine GAD65 under the control of the Cauliflower Mosaic Virus 35S promoter and the polyadenylation signal from the nopaline synthase gene, was constructed and transferred into tobacco plants by the method of *Agrobacterium*-mediated transformation. Following transformation and selection, transgenic tobacco plants were produced. Inte-

gration of canine GAD65 DNA into the nuclear genome of tobacco was confirmed by PCR (polymerase chain reaction) using canine GAD65 specific primers (not shown). Expression of the transferred canine GAD65 at the protein level was determined by Western blot analysis. As shown in FIG. 7, anti-GAD antibody detected a unique band of the expected molecular weight (65 kDa) on Western blots of total leaf extracts prepared from tobacco plants transformed with canine GAD65. In contrast, the same band could not be detected by Western blot analysis in leaf extracts from vector-minus canine GAD transformed tobacco plants. Mouse GAD67 is shown as a size control and is detected by the anti GAD antibody.

Biological Activity of Plant Cell Culture-Derived Recombinant Canine IL-4.

[0138] The biological activity of plant-derived rcIL-4 was determined by *in vitro* bioassay using the cIL-4-dependent cell line (FIGS. 8A, 8B), TF-1. TF-1 cells are a factor-dependent human erythroleukemic cell line that will proliferate in response to canine IL-4. To perform the assay, 6× histidine-tagged rcIL-4 was purified from transgenic tobacco leaf tissue by chromatography on Ni-NTA agarose and used to induce the proliferation of TF-1 cells in comparison with commercial rcIL-4 standard. As shown here, plant-derived rcIL-4 induced TF-1 cells to proliferate in a dose-dependent fashion in culture medium (RPMI 1640), and was comparable to that of rcIL-4 standard. Moreover, co-incubation of plant rcIL-4 with anti-cIL-4 mAb reduced its ability to stimulate the proliferation of TF-1 cells. Taken together, these results suggest that plant-derived rcIL-4 retains its biological and functional authenticity.

Example C

Expression of Canine GAD65 and Canine IL-4 in Suspension Cultures

Dicot Binary Constructs for Expression in Tobacco Cells

[0139] A dicot binary vector, pDAB2457 (Sequence ID No. 1) for *Agrobacterium*-mediated plant transformation was constructed based on plasmids pDAB771, pDAB773 and pDAB2407. pDAB771 (FIG. 9A) contains the cassava vein mosaic virus promoter described in WO 97/48819 (CsVMV) fused to the 5' UTR from *Nicotiana tabacum* osmotin gene (Plant Mol. Bio. 19:577-588 (1992); patent application US 2005102713) and a chimeric 3' untranslated region consisting of 3' UTRs from the *Nicotiana tabacum* osmotin gene (Plant Mol. Bio. 19:577-588 (1992); patent application US 2005102713) and from *Agrobacterium tumefaciens* plasmid Ti 15955 ORF24 (GenBank accession X00493). Located between the CsVMV promoter and ORF24 3'UTR are unique sites, NcoI and SacI, which were used for inserting genes of interest. pDAB773 (FIG. 9B) contains the RB7 matrix attachment region (MAR) (U.S. Pat. No. 5,773,689; U.S. Pat. No. 5,773,695; U.S. Pat. No. 6,239,328, WO 94/07902, and WO 97/27207) and a transcription unit in which the plant selection marker phosphinothricin acetyl transferase (PAT) (U.S. Pat. Nos. 5,879,903; 5,637,489; 5,276,268; and 5,273,894) is driven by the AtUbi10 promoter (Plant J. 1997. 11(5):1017; Plant Mol. Biol. 1993. 21(5):895; Genetics. 1995. 139(2): 921) and flanked, downstream by AtuORF1 3' UTR (U.S. Pat. No. 5,428,147; Plant Molecular Biology. 1983. 2:335; GenBank accession X00493). A unique NotI site, located

between the RB7 MAR gene and the plant AtUbi10 promoter, was used for cloning gene fragments containing the CsVMV promoter, gene of interest, and ORF24 3'UTR. A basic binary vector, pDAB2407 (FIG. 9C) allows for AgeI/AgeI ligation of the genes of interest and selectable marker expression cassettes between the T-DNA borders of the *Agrobacterium* binary vector.

[0140] The IL-4 dicot binary vector, pDAB2457 (FIG. 9D), encodes a canine interleukin-4 protein with endoplasmic reticulum (ER) targeting (native) and ER retention signals (Sequence ID NO. 2, Table B). More specifically, the plant transcription unit includes: T-DNA Border B/RB7 MAR v3/CsVMV promoter v2-Nt Osm 5' UTR v3/IL-4 v2-KDEL/Nt Osm 3' UTR v3-Atu ORF24 3' UTR v2::AtUbi10 promoter v2/PAT v3/AtuORF1 3' UTR v3::T-DNA Border A. The chemically synthesized IL-4 gene contained in DASPICO13 was obtained from PICOSCRIPT in Stratagene's Bluescript vector. A modified version of the gene was produced using PCR and included the IL-4 gene flanked by a 6 histidine tag and an ER retention signal (KDEL). The NcoI/SacI fragment was then inserted into pDAB771 plasmid at the NcoI and SacI sites, resulting in intermediate vector pDAB2455 (FIG. 9E). The CsVMV promoter expression cassette containing IL4 v2-KDEL/ORF24 3'UTR was removed from pDAB2455 with NotI and was ligated in the NotI site of pDAB773, downstream of the RB7 MARv3 gene and upstream of the AtUbi10 promoter v2/PAT v3/AtuORF1 3'UTR selectable marker cassette forming the plant transcription unit (PTU) in intermediate vector pDAB2456 (FIG. 9F). The PTU components were then excised from pDAB2456 using AgeI digestion and ligated in reverse orientation at the AgeI site of pDAB2407 which resulted in the final dicot binary vector, pDAB2457, where the PTU cassette is flanked by T-DNA borders A and B.

Gateway™ Dicot Binary Construct

[0141] Invitrogen's Gateway™ Technology was used for constructing vectors for expression of cGAD65 in tobacco cells. Both the destination and donor vectors were made following Invitrogen's Gateway™ Technology protocol. One destination vector, pDAB3736 (FIG. 9G), and a donor vector, pDAB3741 (FIG. 8), were used to create the cGAD65 binary construct.

[0142] Destination vector, pDAB3736, was derived from pDAB2407 and contains attR sites, which recombine with an entry clone in an LR clonase reaction to generate an expression clone (Invitrogen Gateway Technology). It also contains multiple copies of Border A and Border B of the binary vector. Within the border regions, there are an RB7 matrix attachment region (MAR) and Gateway™ cloning sites attR1 and attR2. Entry vector, pDAB3731 (FIG. 9H) contains the attL sites which are used to clone gene fragments that do not contain att sites to generate entry clones (Invitrogen Gateway Technology). pDAB3931 contains the CsVMV v2 promoter and ORF24 3'UTR v1 cassette. Located between the promoter and UTR are NcoI and SacI sites, where the gene of interest is inserted. The cassette is flanked by Gateway™ cloning sites attL1 and attL2.

[0143] Gateway™ GAD655 binary vector, pDAB3748 (FIG. 9; Sequence ID NO.3, Table C), contains the PTU cassette: T-DNA Border B:: RB7 MAR v3::CsVMV promoter v2/cGAD v2/Atu ORF24 3' UTR v2::AtUbi10 promoter v2/PAT v3/Atu ORF1 3' UTR v3:: Multiple T-DNA Border A. The chemically synthesized cGAD65 gene (native cGAD65-Sequence ID NO. 4; modified cGAD65-Sequence

ID NO. 5), which was optimized for plant expression, was excised from pDASPICO27 using NcoI and SacI. The cGAD65 fragment was ligated into the NcoI/SacI sites of pDAB3931 to form the entry clone, pDAB3741. pDAB3741 was transferred into pDAB3736 using LR Clonase to form pDAB3748.

Monocot Constructs for Expression in Rice Cells

[0144] Rice transformation was done using purified DNA fragments. The expression cassette was flanked by FspI sites to allow for purification of the expression cassette from the vector backbone. The expression cassette in pDAB2453 (FIG. 9K; Sequence ID NO.6, Table F) was comprised of a promoter from the maize ubiquitin gene (ZmUbi1 v2; Plant Mol. Biol. 1994. 26(3). 1007; U.S. Pat. No. 5,614,399), modified to remove an NcoI site, and the 3' UTR region from a maize peroxidase gene (ZmPer5 3' UTR v2; U.S. Pat. No. 6,699,984). The selectable marker gene cassette included in pDAB2453 was the PAT gene (described above) flanked by the rice actin gene promoter (OsAct1 v2; Mol. Gen. Genet. 1991. 231:150; GenBank accessions S44221 and X63830;), modified to remove a SacI site and the 3' UTR from a maize lipase gene (ZmLip 3' UTR v2; GenBank accession L35913.). The chemically synthesized IL-4 gene was contained in DASPICO13 and was obtained from PICOSCRIPT in Stratagene's Bluescript vector. A modified version of the gene was produced using PCR to create the IL-4 gene flanked by a 6 histidine tag (Sequence ID NO.7, Table 6). The NcoI/SacI fragment from the PCR product was then inserted into pDAB4005 (FIG. 9L) at the NcoI and SacI sites, resulting in intermediate vector pDAB2451 (FIG. 9M). The ZmUbi1 promoter expression cassette containing IL4 v2-KDEL/ZmPer5 3'UTR was removed from pDAB2451 with NotI and was ligated into the NotI site of pDAB8504 (FIG. 9N), upstream of the OsAct1 promoter v2/PAT v3/ZmLip 3' UTR v2 selectable marker cassette forming the plant transcription unit (PTU) in pDAB2453. The PTU components were then excised from pDAB2453 using FseI digestion and purified for rice transformation experiments.

Production of T-309 Rice Suspensions Stably Transformed with pDAB2453 containing the cIL-4 Gene

[0145] Starting material for rice transformations was T309 rice suspension cells maintained in liquid AA media (AA Custom Mix PhytoTechnology Laboratories L.L.C. catalog number CM024), by transferring 8 ml of settled cell volume and 28 ml of conditioned media (media recovered from suspension cultures) into 80 ml of fresh AA cell culture media in 500 ml flasks every three and a half days. Flasks were maintained on a rotary shaker at 28° C. and 125 rpm. WHISKERS™ experiments were initiated by transferring 9 ml of settled cell volume and 27 ml of conditioned media into 80 ml of fresh AA liquid media. Two 500-ml flasks were maintained on a rotary shaker at 125 rpm and 28° C. for 24 hours prior to treatment.

[0146] On the day of treatment, the cells were given an osmotic pre-treatment of 30 minutes by drawing off the conditioned media and replacing it with 72 ml of AA liquid media containing 0.25 M sorbitol and 0.25 M mannitol. Following osmotic treatment, the two flasks were pooled into a sterile 250 ml IEC centrifuge bottle (Fisher Scientific catalog number 05-433B). Once the cells had settled, the osmotic media was removed leaving approximately 50 ml of settled cells and media at the bottom of the bottle. Osmotic media was saved to be used during recovery described below.

[0147] Whiskering was carried out by adding 8100 µl of freshly prepared 5% Whiskers Suspension (Silar SC-9, Advanced Composite Materials Corp, Greer, S.C.) and 170

µg of plasmid DNA, pDAS2453. The bottle was placed in the modified paint mixer (Red Devil Equipment Co., Minneapolis, Minn.) and agitated on high for 10 seconds after which cells were returned to a 1 L flask with conditioned media and 208 ml of fresh AA liquid media was added. Cells were allowed to recover for 2 hours on a rotary shaker at 125 rpm and 28° C.

[0148] Following recovery, 1 ml aliquots of cell suspension were evenly dispensed on sterile, 55 mm number 4 filter paper discs (Whatman International Ltd.) resting on a 60x20 mm Petri dishes containing semi-solid AA media (AA Custom Mix PhytoTechnology Laboratories L.L.C. catalog number CM024 plus 2.5 g/L Gelrite, Sigma-Aldrich catalog number G 9110) and incubated at 28° C. in the dark for three days. After three days, filters with cells were transferred to fresh semi-solid D2[-]P media (N6 Salts catalog number C1416 PhytoTechnology Laboratories, MS/N6 vitamins, 1 g/L tryptophan, 30 g/L sucrose, 5 mg/L 2,4-D, 2.5 g/L Gelrite, Sigma-Aldrich catalog number G1910, and 3.0 mg/L Herbiace Meiji, Toyoko, Japan) and incubated in the dark at 28° C. for 2 weeks. Filters were transferred to fresh D2[-]P media every 2 weeks until isolates appeared. Calli were placed on semi-solid AA media containing 5 mg/L Herbiace and sub-cultured every 2 weeks. Expression analysis was performed on selected events.

Production of Transgenic *Nicotiana tabacum* Events Transformed with pDAB2457 containing the cIL-4 Gene

[0149] Four days prior to transformation, a 1 week old NT-1 culture was sub-cultured to fresh medium by adding 2 ml of the NT-1 culture or 1 ml of packed cells into 40 ml NT-1 B media. The sub-cultured suspension was maintained in the dark at 25±1° C. on a shaker at 125 rpm.

NT-1 B Medium	
Reagent	Per liter
MS salts (10X)	100 ml
MES	0.5 g
Thiamine-HCl (1 mg/ml)	1 ml
Myo-inositol	100 mg
K ₂ HPO ₄	137.4 mg
2,4-D (10 mg/ml)	222 µl
Sucrose	30 g
pH to 5.7 ± 0.03	

Thiamine-HCl (1 mg/ml)(1 liter)

Thiamine HCl (Vit B1) - 0.1 g

2,4-D (10 mg/ml)

Stock solution purchased from Phytotechnology Laboratories

[0150] A 50% glycerol stock of *Agrobacterium tumefaciens* containing the expression vector of interest was used to initiate a liquid culture directly by adding 20-500 µl of the bacteria to 30 ml YEP liquid containing 50 mg/L spectinomycin. The bacterial culture was incubated in the dark at 28° C. in an incubator shaker at 150-200 rpm.

YEP Medium	
Reagent	Per liter
Yeast extract	10 g
Peptone	10 g

-continued	
YEP Medium	
Reagent	Per liter
NaCl	5 g
Sucrose	10 g

Four milliliters of the tobacco suspension was transferred into each of 10, 100×25 mm Petri plates. For the treated plates, 100 μ l of *Agrobacterium* suspension at $OD_{600}=1.5\pm0.2$ was added to each of the 9 plates, keeping one plate as an untreated control. The plates were swirled to mix, wrapped in parafilm and cultured in the dark at $25\pm1^\circ\text{C}$. without shaking.

[0151] Following the co-cultivation, all liquid was removed and the cells were resuspended in 8 ml NTC medium (NT-1 medium containing 500 mg/L carbenicillin, added after autoclaving). One milliliter aliquots of suspension were distributed to each of 8 Petri plates (100×25 mm) containing NTC+B10 medium (NTC medium solidified with 8 g/l TC Agar supplemented with 10 mg/l bialaphos, added after autoclaving). All selection plates, either wrapped with parafilm or left unwrapped, were maintained in the dark at 28°C . Before wrapping, liquid was removed from any plates that were excessively wet. After 2 to 6 weeks, putative transformants appeared as small clusters of callus on a background of dead, non-transformed cells. They were selected and transferred to fresh NTC+B10. The plates were left unwrapped and cultured in the dark at $28\pm1^\circ\text{C}$. Portions of each putative transformant was collected for analysis.

Extraction of Callus Samples

[0152] For western analysis, callus samples are extracted directly in SDS-PAGE gel loading buffer. Two hundred microliters of 2× Laemmli sample buffer (with DTT as the reducing agent) was added to 200 μ l of callus tissue. Two steel BBs (Daisy 4.5 mm) were added to each tube and the tubes were shaken for 2 minutes in a Klecko tissue disrupter. After heating for 5-10 minutes at 95°C ., the tubes were centrifuged in a microfuge for 10 minutes. The samples were loaded on gels for western analysis.

Western Analyses of Transgenic Callus Events

[0153] Samples for SDS-PAGE were prepared as above for whole cell extracts or by adding loading buffer (4× Laemmli Sample Buffer with DTT) and heating for 5 minutes ($90-100^\circ\text{C}$.). Gels (Invitrogen NuPAGE 4-12% Bis-Tris Gel) were run using MES Running Buffer (Invitrogen catalog number NP0002-02). Molecular weight standards (SeeBlue Plus2, MagicMark XP SeeBlue Plus2; catalog numbers LC5925 and LC5602, respectively) and appropriate volume of samples were loaded. The gels were run at 200V for 30-45 minutes. The membranes (0.2 μ m nitrocellulose membrane; Invitrogen catalog number LC2000) and pads were soaked for 10-30 min in 10% Methanol Transfer Buffer (NuPAGE Transfer Buffer catalog number NP0006).

[0154] The blot module was assembled according to manufacturer directions and blots were transferred at 30V for approximately 1 hour. After transfer, the membranes were rinsed with water and blocked for at least 30 minutes at room temperature with agitation in block solution (WesternBreeze Blocker/Diluent Invitrogen catalog number WB7050). The

blots were incubated at least 1 hr in primary antibody in block solution. The membrane was washed 3 times for 5 min each in wash solution (WesternBreeze Wash Solution catalog number WB7003). Treatment with the secondary antibody was similar except the incubation was for at least 30 min. The membrane was washed 3 times for 5 min each in wash solution followed by 2 washes for 2 min each in water prior to adding substrate.

[0155] For IL-4 western blots, the standard was recombinant canine IL-4 (R&D Systems catalog number 754-CL); the primary antibody (diluted to 1 μ g/ml) was anti-canine IL-4 antibody (R&D Systems catalog number AF 754); the secondary antibody was rabbit anti-goat IgG HRP conjugated (Sigma catalog number A5420) diluted 1:5000. Western immunodetection was done using WesternBreeze Kit (Invitrogen catalog number WB7050) and the Pierce SuperSignal West Pico Luminol Enhancer and Stable Peroxide Solution mixed in equal parts (Pierce catalog number 34080) for detection. The blots were exposed to X-ray film to determine IL-4 expression in the transgenic calli.

[0156] For GAD65 western blots, the standard was rhGAD65 (Diamyd Diagnostics catalog number 10-65702-01); the primary antibody was anti-GAD65 (Sigma catalog number G1166) diluted 1:2000; the secondary antibody was goat anti-mouse IgG AP (KPL catalog number 075-1806) diluted 1:1000. The western immunodetection was done using WesternBreeze Kit (Invitrogen catalog number WB7050) and the NBT/BCIP Phosphatase Substrate (KPL catalog number 50-81-08) for detection. The blots were exposed to substrate for 5-10 minutes to determine GAD65 expression in the transgenic calli.

[0157] Western analysis demonstrated that canine cIL-4 is expressed in both rice and tobacco cells (FIGS. 10 and 11). As evident from its higher molecular weight relative to the cIL-4 reference protein, the transgenic cIL-4 appears to be post-translationally modified. Canine GAD65 targeted to the cytoplasm is expressed in tobacco cells (FIG. 12). The molecular weight of the transgenic protein is similar to or higher than the cGAD65 reference. Degradation products are also apparent in the western blot.

Characterization of cIL-4

[0158] Transgenic cIL-4 was further characterized by extraction of the protein from transgenic tobacco calli. Tissue was ground in liquid nitrogen and ~10 volumes per weight (ml/g tissue) of 2×PBST (Sigma P3563), 1 M urea, 10% glycerol, 2 mM imidazole, 1 mM PMSF, and 1% protease cocktail inhibitor (Sigma P9599) was added. The suspension was stirred at 4°C . for 30 min. After clarification by centrifugation followed by filtration, the solution was loaded on a Hi-Trap Nickel column (GE Healthcare 17-5247-01) and allowed to recirculate for ~2 hrs at 2.5 mL/min. The column was washed with 2×PBST, 40 mM imidazole, pH 8.4 and the bound protein was eluted with 20 mM NaH_2PO_4 , 500 mM NaCl and 500 mM imidazole, pH 7.4. The fractions containing cIL-4, as determined by western blot analysis, were combined and loaded on a 100 ml Superose 6 16/50 sizing column (GE Healthcare 17-0489-01) column. Protein was eluted in PBS, pH 7.4 and tested in the in vitro IL-4 activity assay. Samples of the fractions were separated by SDS-PAGE and the major protein band eluted was analyzed by MALDI-TOF to confirm its identity as IL-4 (data not shown). cIL-4 produced in transgenic tobacco callus was purified as described above. The chromatograph of the Hi-Trap Nickel column is shown in FIG. 13, with the fractions retained for further purification. SDS-PAGE analysis of the fractions eluted from the Superose 6 column identified a major protein band (arrow) that corresponded to cIL-4 as determined by western blot and MALDI-TOF analysis.

TABLE A

Sequence ID 1: pDAB2457

ccggtaggatccggtgagtaatatgtacggctaagagcgaatttgccctgtagacctcaattgcgagctttctaatttc
aaactattcgggcctaacttttgggtgatgatgctgactggcaggatataaccgttgtaatttgagctcgtgtgaataa
gtcgtgtgtatgtttgtttgatgtttctgttgagtgacagccatttcacggacaagtcggctagattgatttagccctg
atgaactgccgaggggaagccatcttgagcgcggaatgggaatggatcgaacggggagcacaggatgacgcctaac
aattcattcaagccgacaccgcttcgcgggcgcggttaattcaggagttaaacatcatgaggggaagcgggtgatcgccga
agtatcgactcaactatcagaggtagttggcgctcatcgagcgccatctcgaaccgacgttgctggccgtacatttgtacg
gtccgcagtggtggcgccctgaagccacacagtgatattgatttgctggttacggtgaccgtaaggcttgatgaaac
aacggcgcgagcttgatcaacgaccttttgaaacttcggcttccccctggagagagcgagattctccgctgtagaag
tcaccattgttgtgcacgacgacatcattccgtggcggttatccagctaagcgcgaactgcaatttgagaatggcagcgc
aatgacattcttgaggtatcttcgagccagccagatcgacattgatctggctatcttgctgacaaaagcaagagaaca
tagcgttgcttggttaggtccagcggcgagggaactcttgatccggttcctgaacaggatctatttgaggcgctaaatg
aaaccttaacgctatggaactcgccgcgcgactggcggtggcgatgagcgaatgtagtgcttacgtgtgtccgcatttgg
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TABLE A-continued

Sequence ID 1: pDAB2457

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TABLE A-continued

Sequence ID 1: pDAB2457

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TABLE A-continued

Sequence ID 1: pDAB2457

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attgagatttttaacgcggcccatgatcaca

TABLE B

Sequence ID 2: canine IL-4 optimized for plant
expression with an ER retention signal and a 6
histidine tag

atgggcctcacatcacaaactgattccgactcttgtctgtctccttgccctcacctccacatttgttcatggacacaaacttcaat
atcaccatttaaggagataatacaaatgttgaaacttttgacagcaaggaatgatagttgcatggagctgactgtgaagg
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TABLE C

Sequence ID 3: pDAB3748

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TABLE C-continued

Sequence ID 3: pDAB3748

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TABLE C-continued

Sequence ID 3: pDAB3748

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TABLE C-continued

Sequence ID 3: pDAB3748

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TABLE C-continued

Sequence ID 3: pDAB3748

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TABLE D

Sequence ID 4: native canine GAD65

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TABLE D-continued

Sequence ID 4: native canine GAD65
<p> tggagccatctccaacatgtatgccatgctcattgccagattcaagatgtttccagaagtcaaagagaaaggcatggca gctgttccgaggctcattgcattcacatcagaacacagtcatttcagcctcaagaaaggagctgcagcactgggcatag gcacagacagtgatgatactcatcaagtgtgatgagagaggcaagatgggtccctctgatcttgaacgtcgaatccttgaa gccaaacagaaaggcttgttccctctcttggtgtcagccactgctggcacaactgtgtacgggtgcctttgatcctctcttg ctgttgctgatctctgaagaagtacaagatctggatgcattgtggatgctgcctggggtggagggttgctcatgtcaagg aaacacaagtggaagctgagtggtgtgaacgtgccaaactctgtcacatggaatccacacaagatgatgggtgttcctc tccaatgttctgctctctctgtgcgtgaagaggattgatgcagtcagcaatcagatgcagcctcctacctcttccagcaa gacaaacactatgatctgtcctatgacactggagacaaagccttgcaagtgtggacgacatgttgatgtcttcaagctctg gctcatgtggagagccaaagggaccactgggtttgaggctcacatagacaagtgttggagcttgagagtagctgtac agcattatcaagaatcgagaaggctacgagatgggtttgatgggaagccacacaccaatgtctgcttctggtagt tcctccgtcattgagggttctggaagacaacgaagagaggatgaacagactctcaaaggtggcaccagtgatcaaggc aaggatgatggagtagggaccacaatggtagctaccagccactgggagacaagtgaaactctttcgaaatggtagtc tccaatcctgctgcaactcatcaagacattgactttctcattgaagagattgaagacttggaacaagatctt </p>

TABLE E

Sequence ID 5: canine GAD65 optimized for plant expression
<p> atggcatcaccgggaagcggtttctggctcttggctccgaggacggatctggggaccctgagaatccatcaactgcac gagcatgggtgccaaagtgcacagaagttcactgggtggcatgggaacaaactttgtgctctcttgtaggagatgctgag aaaccagcagaatctgggtggaagtgaaccaccagagctaccagtaggaaagctgcctgtgcctgcaatcagaaacc ctgcagttgtccaaagctgaagtcaactacgcattcctgcatgccactgatctggtgccagcctgcgatggagaacgtcc gactctggcattccttcaagatgtcattggacattctcttgcatgtgttggaagtcatctgacagatctacaaagtattg actttcactatccgaatgagcttctccaagagtacaactgggagttggcagatcaacctcagaatcttgaagagatactg atgcattgccaaacaaactctcaagatgtcatcaagactggtagctcctcgttacttcaatcagttgtccactgggttgaca tgggttggtcttgacagtgattgggtgacatccactgccaaacaaacatgttcacatgatgagatagctcctgtcttctgtctg ctggagtagtgtagactcaagaagatgagggagatcatggctggcctgggtggctctggagatgggattctctctcctgg tggagccatctccaacatgtatgccatgctcattgccagattcaagatgtttccagaagtcaaagagaaaggcatggca gctgttccgaggctcattgcattcacatcagaacacagtcatttcagcctcaagaaaggagctgcagcactgggcatag gcacagacagtgatgatactcatcaagtgtgatgagagaggcaagatgggtccctctgatcttgaacgtcgaatccttgaa gccaaacagaaaggcttgttccctctcttggtgtcagccactgctggcacaactgtgtacgggtgcctttgatcctctcttg ctgttgctgatctctgaagaagtacaagatctggatgcattgtggatgctgcctggggtggagggttgctcatgtcaagg aaacacaagtggaagctgagtggtgtgaacgtgccaaactctgtcacatggaatccacacaagatgatgggtgttcctc tccaatgttctgctctctctgtgcgtgaagaggattgatgcagtcagcaatcagatgcagcctcctacctcttccagcaa gacaaacactatgatctgtcctatgacactggagacaaagccttgcaagtgtggacgacatgttgatgtcttcaagctctg gctcatgtggagagccaaagggaccactgggtttgaggctcacatagacaagtgttggagcttgagagtagctgtac agcattatcaagaatcgagaaggctacgagatgggtttgatgggaagccacacaccaatgtctgcttctggtagt tcctccgtcattgagggttctggaagacaacgaagagaggatgaacagactctcaaaggtggcaccagtgatcaaggc </p>

TABLE E-continued

Sequence ID 5: canine GAD65 optimized for plant expression
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TABLE F

Sequence ID 6: pDAB2453
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TABLE F-continued

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TABLE F-continued

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TABLE F-continued

Sequence ID 6: pDAB2453
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TABLE G

Sequence ID 7: canine IL-4 optimized for plant expression with a 6 histidine tag
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[0159] Although preferred embodiments of the invention have been described herein in detail, it will be understood by those skilled in the art that variations may be made thereto without departing from the spirit of the invention or the scope of the appended claims.

1. A method for the treatment of new onset Type I diabetes in a mammal or pre-Type I diabetic mammal, the method comprising:

- (a) administering anti-T cell therapy to said mammal; and
- (b) administering an autoantigen composition comprising an optional mucosal antigen, wherein (a) and (b) are administered concurrently or sequentially.

2. The method of claim 1, wherein said anti-T cell therapy is an immunosuppressant agent that targets T cells.

3. The method of claim 2, wherein said immunosuppressant agent is selected from the group consisting of monoclonal antibodies targeting T cell surface antigens, polyclonal antibodies targeting T cell surface antigens, cyclosporine, methotrexate, azathioprine and combinations thereof.

4. The method of claim 3, wherein said immunosuppressant agent is a monoclonal antibody selected from the group consisting of anti CD3, anti CD2, anti CD4 anti CD7, anti CD8, anti CD25, anti CD28, anti alpha 4 beta 1 integrin, anti alpha 4 beta 7 integrin and combinations thereof.

5. The method of claim 4, wherein said immunosuppressant is an anti CD3 monoclonal antibody.

6. The method of claim 1, wherein said autoantigen is selected from the group consisting of a GAD isoform, GAD polypeptide, insulin and combinations thereof.

7. The method of claim 6, wherein said autoantigen is a GAD isoform selected from the group consisting of GAD65, GAD67 and mixtures thereof.

8. The method of claim 1, wherein said mucosal antigen is an immunoregulatory cytokine.

9. The method of claim 8, wherein said immunoregulatory cytokine is an interleukin.

10. The method of claim 9, wherein said interleukin is selected from the group consisting of IL-1, IL2, IL-3, IL4, IL5, IL6, IL-7, IL-8, IL9, IL-10, IL-12, IL-13, IL15, IL18 and mixtures thereof.

11. The method of claim 10, wherein said interleukin is IL-4.

12. The method of claim 10, wherein said interleukin is IL10.

13. The method of claim 2, wherein said immunosuppressant agent is administered intravenously to said mammal for up to about 10 days.

14. The method of claim 13, wherein immunosuppressant agent is administered intravenously to said mammal for up to about 5 to 7 days.

15. The method of claim 13, wherein said immunosuppressant agent is administered at dosages of up to about 10 μ /kg to up to about 100 μ /kg body weight.

16. The method of claim 1, wherein said autoantigen and optional mucosal antigen composition is administered orally or to a mucosal surface or parentally.

17. The method of claim 16, wherein said autoantigen and optional mucosal antigen composition is provided within a transgenic plant material.

18. The method of claim 17, wherein said transgenic plant material is selected from the group consisting of potato, tomato, alfalfa, canola, rice, tobacco, maize, algae, safflower, moss and bryophyte.

19. The method of claim 17, wherein said transgenic plant material is selected from the group consisting of plant tissue, plant leaves, plant tubers, plant stems, plant extracts, plant slurries, plant cell cultures and combinations thereof.

20. The method of claim 17, wherein said composition is administered orally.

21. The method of claim 16, wherein said autoantigen and optional mucosal antigen is provided in an amount of up to about 1 mg/kg to up to about 1000 mg/kg.

22. The method of claim 16, wherein said autoantigen and optional mucosal antigen is provided in an amount of more than about 1000 mg/kg.

23. The method of claim 21, wherein said autoantigen and optional mucosal antigen is provided in an amount of up to about 1 mg/kg to up to about 100 mg/kg.

24. The method of claim 1, wherein (a) and (b) are administered concurrently.

25. The method of claim 1, wherein (a) and (b) are administered sequentially.

26. The method of claim 1, wherein (a) and (b) are administered concurrently followed by further administration of (b).

27. The method of claim 26, wherein said further administration of (b) is done for an extended period of time.

28. The method of claim 27, wherein said extended period of time is up to about the lifespan of the mammal.

29. The method of claim 1, wherein said mammal is a human.

30. The method of claim 1, wherein said mammal is a companion animal selected from the group consisting of dogs, cats and horses.

31. The method of claim 29, wherein said human has new onset Type I diabetes.

32. The method of claim 29, wherein said human is pre-Type I diabetic.

33. A method for treating Type I diabetes in a mammal or for treating pre-type I diabetic mammals, the method comprising:

- (a) administering an effective dose of anti-T cell antibodies to said human; and

- (b) administering an effective dose of an autoantigen to said mammal, wherein (a) and (b) are administered at the same time or sequentially for an effective time period, or (a) and (b) are administered at the same time and (b) is further administered alone for a longer time period.

34. A method for treating Type I diabetes in a human, or for treating pre-type I diabetic humans, the method comprising:

- (a) administering an effective immunosuppressive dose of anti-T cell antibodies to said humans; and

- (b) administering an effective immunosuppressive dose of a transgenic plant material to said mammal, said transgenic plant material containing at least one autoantigen and optionally at least one immunoregulatory cytokine; wherein said administering of (a) and (b) is done concurrently or sequentially.

35. The method of claim 34, wherein said anti-T cell antibodies are polyclonal antibodies.

36. The method of claim 34, wherein said anti-T cell antibodies are monoclonal antibodies.

37. The method of claim 36, wherein said monoclonal antibody is selected from the group consisting of anti CD3, anti CD2, anti CD4, anti CD7, anti CD8, anti CD25 anti CD28, anti alpha 4 beta 1 integrin, anti alpha 4 beta 7 integrin and combinations thereof.

38. The method of claim 37, wherein said monoclonal antibody is an anti CD3 monoclonal antibody.

39. The method of claim 34, wherein said autoantigen is selected from the group consisting of GAD isoform, GAD polypeptide, insulin and combinations thereof.

40. The method of claim 39, wherein said autoantigen is a GAD isoform selected from the group consisting of GAD65, GAD67 and mixtures thereof.

41. The method of claim 34, wherein said immunoregulatory cytokine is an interleukine.

42. The method of claim 41, wherein said interleukin is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-18 and mixtures thereof.

43. The method of claim 42, wherein said interleukin is IL-4.

44. The method of claim 42, wherein said interleukin is IL-10.

45. The method of claim 41, wherein said transgenic plant material is administered orally or to a mucosal surface.

46. The method of claim 45, wherein said transgenic plant material is administered to provide up to about 10 µg/kg to up to about 100 µg/kg body weight of said anti-T cell antibodies.

47. The method of claim 46, wherein said transgenic plant material provides about up to about 1 mg/kg to up to about 1000 mg/kg of autoantigen and optional immunoregulatory cytokine.

48. The method of claim 34, wherein (a) and (b) are administered concurrently.

49. The method of claim 34, wherein (a) and (b) are administered sequentially.

50. The method of claim 34, wherein (a) and (b) are administered concurrently followed by further administration of (b).

51. The method of claim 50, wherein said further administration of (b) is done for an extended period of time.

52. The method of claim 51, wherein said extended period of time is up to about the lifespan of the mammal.

53. A method for reversal of Type I diabetes in a human or companion animal, said method comprising:

(a) administering a therapeutically effective amount of anti-CD3 monoclonal antibody to said human or animal; and

(b) administering a therapeutically effective amount of a transgenic plant material containing one or more GAD autoantigens together with IL-4,

wherein (a) is first administered to said human or animal.

54. The method of claim 53, wherein (a) and (b) are administered concurrently.

55. The method of claim 53 wherein (b) is further administered for an extended period of time.

56. An IL-4 nucleotide sequence optimized for plant expression.

57. The sequence of claim 56, wherein said optimization is the addition of a histidine tag.

58. The sequence of claim 56, wherein said optimization is the addition of an ER retention signal and histidine tag.

59. The sequence of claim 56, wherein said nucleotide sequence is selected from the group consisting of SEQ ID NO. 2 and SEQ ID NO. 7.

60. The sequence of claim 56, wherein said sequence is a canine sequence.

61. A canine GAD65 nucleotide sequence of SEQ ID NO. 4.

62. The nucleotide sequence of claim 61 wherein said sequence is further optimized for plant expression.

63. The nucleotide sequence of claim 62, wherein said optimized sequence is represented by SEQ ID NO. 5.

64. A vector for cell transformation, said vector selected from the group consisting of SEQ ID NO. 1, SEQ ID NO. 3 and SEQ ID NO. 6.

65. A vector selected from the group consisting of pDAB771; pDAB773; pDAB2407; pDAB2457; pDAB2455; pDAB2456; pDAB3736; pDAB3741; pDAB3731; pDAB3748; pDAB2453; PDAB4005; pDAB2451; and pDAB8504.

66. A composition comprising a mixture of anti-CD3 antibodies and a preparation that contains at least one autoantigen and an immunoregulatory cytokine.

67. A composition comprising a mixture of anti-CD3 antibodies and a transgenic plant material that contains at least one autoantigen and an immunoregulatory cytokine.

68. The use of a composition comprising anti-T cell antibodies, autoantigen and optional mucosal antigen in the manufacture of a medicament for the treatment of Type I diabetes in a mammal.

69. A method for the diagnosis of Type I diabetes in a mammal, the method comprising detecting in a sample from said mammal the presence of anti-GAD antibodies, such detection being an early indicator of the development or the risk of development of Type I diabetes in the mammal.

70. The method of claim 69, wherein the anti-GAD antibodies are canine antibodies.

* * * * *

EXHIBIT 25

Amendments to the Claims:

This listing of claims will replace all prior versions and listings of claims in the application:

Listing of Claims:

Claim 1 (Original): A method for the treatment of new onset Type I diabetes in a mammal or pre-Type I diabetic mammal, the method comprising:

- (a) administering anti-T cell therapy to said mammal; and
- (b) administering an autoantigen composition comprising an optional mucosal antigen, wherein (a) and (b) are administered concurrently or sequentially.

Claim 2 (Original): The method of claim 1, wherein said anti-T cell therapy is an immunosuppressant agent that targets T cells.

Claim 3 (Original): The method of claim 2, wherein said immunosuppressant agent is selected from the group consisting of monoclonal antibodies targeting T cell surface antigens, polyclonal antibodies targeting T cell surface antigens, cyclosporine, methotrexate, azathioprine and combinations thereof.

Claim 4 (Original): The method of claim 3, wherein said immunosuppressant agent is a monoclonal antibody selected from the group consisting of anti CD3, anti CD2, anti CD4, anti CD7, anti CD8, anti CD25, anti CD28, anti alpha 4 beta 1 integrin, anti alpha 4 beta 7 integrin and combinations thereof.

Claim 5 (Original): The method of claim 4, wherein said immunosuppressant is an anti CD3 monoclonal antibody.

Claim 6 (Original): The method of claim 1, wherein said autoantigen is selected from the group consisting of a GAD isoform, GAD polypeptide, insulin and combinations thereof.

Claim 7 (Original): The method of claim 6, wherein said autoantigen is a GAD isoform selected from the group consisting of GAD65, GAD67 and mixtures thereof.

Claim 8 (Original): The method of claim 1, wherein said mucosal antigen is an immunoregulatory cytokine.

Claim 9 (Original): The method of claim 8, wherein said immunoregulatory cytokine is an interleukin.

Claim 10 (Original): The method of claim 9, wherein said interleukin is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-18 and mixtures thereof.

Claim 11 (Original): The method of claim 10, wherein said interleukin is IL-4.

Claim 12 (Original): The method of claim 10, wherein said interleukin is IL-10.

Claim 13 (Original): The method of claim 2, wherein said immunosuppressant agent is administered intravenously to said mammal for up to about 10 days.

Claim 14 (Original): The method of claim 13, wherein immunosuppressant agent is administered intravenously to said mammal for up to about 5 to 7 days.

Claim 15 (Currently Amended): The method of claim 13, wherein said immunosuppressant agent is administered at dosages of up to about 40 μ /kg 10 μ /kg to up to about 400 μ /kg 100 μ /kg body weight.

Claim 16 (Currently Amended): The method of ~~claim 4 or 10~~ claim 1, wherein said autoantigen and optional mucosal antigen composition is administered orally or to a mucosal surface or parentally.

Claim 17 (Currently Amended): The method of claim 16, wherein said ~~autoantigen and~~ autoantigen and optional mucosal antigen composition is provided within a transgenic plant material.

Claim 18 (Original): The method of claim 17, wherein said transgenic plant material is selected from the group consisting of potato, tomato, alfalfa, canola, rice, tobacco, maize, algae, safflower, moss and bryophyte.

Claim 19 (Original): The method of claim 17, wherein said transgenic plant material is selected from the group consisting of plant tissue, plant leaves, plant tubers, plant stems, plant extracts, plant slurries, plant cell cultures and combinations thereof.

Claim 20 (Original): The method of claim 17, wherein said composition is administered orally.

Claim 21 (Currently Amended): The method of claim 16, wherein said autoantigen and optional mucosal antigen is provided in an amount of up to about ~~4mg/kg~~ 1 mg/kg to up to about 1000 mg/kg.

Claim 22 (Original): The method of claim 16, wherein said autoantigen and optional mucosal antigen is provided in an amount of more than about 1000 mg/kg.

Claim 23 (Currently Amended): The method of claim 21, wherein said autoantigen and optional mucosal antigen is provided in an amount of up to about 4mg/kg 1 mg/kg to up to about 100 mg/kg.

Claim 24 (Original): The method of claim 1, wherein (a) and (b) are administered concurrently.

Claim 25 (Original): The method of claim 1, wherein (a) and (b) are administered sequentially.

Claim 26 (Original): The method of claim 1, wherein (a) and (b) are administered concurrently followed by further administration of (b).

Claim 27 (Original): The method of claim 26, wherein said further administration of (b) is done for an extended period of time.

Claim 28 (Original): The method of claim 27, wherein said extended period of time is up to about the lifespan of the mammal.

Claim 29 (Original): The method of claim 1, wherein said mammal is a human.

Claim 30 (Original): The method of claim 1, wherein said mammal is a companion animal selected from the group consisting of dogs, cats and horses.

Claim 31 (Original): The method of claim 29, wherein said human has new-onset Type I diabetes.

Claim 32 (Original): The method of claim 29, wherein said human is pre-Type I diabetic.

Claim 33 (Original): A method for treating Type I diabetes in a mammal or for treating pre-type I diabetic mammals, the method comprising:

(a) administering an effective dose of anti-T cell antibodies to said human; and

(b) administering an effective dose of an autoantigen to said mammal, wherein (a) and (b) are administered at the same time or sequentially for an effective time period, or (a) and (b) are administered at the same time and (b) is further administered alone for a longer time period.

Claim 34 (Original): A method for treating Type I diabetes in a human, or for treating pre-type I diabetic humans, the method comprising:

(a) administering an effective immunosuppressive dose of anti-T cell antibodies to said humans; and

(b) administering an effective immunosuppressive dose of a transgenic plant material to said mammal, said transgenic plant material containing at least one autoantigen and optionally at least one immunoregulatory cytokine;

wherein said administering of (a) and (b) is done concurrently or sequentially.

Claim 35 (Original): The method of claim 34, wherein said anti-T cell antibodies are polyclonal antibodies.

Claim 36 (Original): The method of claim 34, wherein said anti-T cell antibodies are monoclonal antibodies.

Claim 37 (Original): The method of claim 36, wherein said monoclonal antibody is selected from the group consisting of anti CD3, anti CD2, anti CD4, anti CD7, anti CD8, anti CD25, anti CD28, anti alpha 4 beta 1 integrin, anti alpha 4 beta 7 integrin and combinations thereof.

Claim 38 (Original): The method of claim 37, wherein said monoclonal antibody is an anti CD3 monoclonal antibody.

Claim 39 (Original): The method of claim 34, wherein said autoantigen is selected from the group consisting of GAD isoform, GAD polypeptide, insulin and combinations thereof.

Claim 40 (Original): The method of claim 39, wherein said autoantigen is a GAD isoform selected from the group consisting of GAD65, GAD67 and mixtures thereof.

Claim 41 (Currently Amended): The method of ~~claim 34, 37, or 39~~
claim 34, wherein said immunoregulatory cytokine is an interleukine.

Claim 42 (Original): The method of claim 41, wherein said interleukin is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL- 8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-18 and mixtures thereof.

Claim 43 (Original): The method of claim 42, wherein said interleukin is IL-4.

Claim 44 (Original): The method of claim 42, wherein said interleukin is IL-10.

Claim 45 (Original): The method of claim 41, wherein said transgenic plant material is administered orally or to a mucosal surface.

Claim 46 (Currently Amended): The method of claim 45, wherein said transgenic plant material is administered to provide up to about ~~40 µg/kg~~ 10 µg/kg to up to about ~~400 µg/kg~~ 100 µg/kg body weight of said anti-T cell antibodies.

Claim 47 (Currently Amended): The method of claim 46, wherein said transgenic plant material provides about up to about ~~4 mg/kg~~ 1 mg/kg to up to about ~~4000 mg/kg~~ 1000 mg/kg of autoantigen and optional immunoregulatory cytokine.

Claim 48 (Original): The method of claim 34, wherein (a) and (b) are administered concurrently.

Claim 49 (Original): The method of claim 34, wherein (a) and (b) are administered sequentially.

Claim 50 (Original): The method of claim 34, wherein (a) and (b) are administered concurrently followed by further administration of (b).

Claim 51 (Original): The method of claim 50, wherein said further administration of (b) is done for an extended period of time.

Claim 52 (Original): The method of claim 51, wherein said extended period of time is up to about the lifespan of the mammal.

Claim 53 (Original): A method for reversal of Type I diabetes in a human or companion animal, said method comprising:

- (a) administering a therapeutically effective amount of anti-CD3 monoclonal antibody to said human or animal; and
 - (b) administering a therapeutically effective amount of a transgenic plant material containing one or more GAD autoantigens together with IL-4,
- wherein (a) is first administered to said human or animal.

Claim 54 (Original): The method of claim 53, wherein (a) and (b) are administered concurrently.

Claim 55 (Original): The method of claim 53, wherein (b) is further administered for an extended period of time.

Claim 56 (Original): An IL-4 nucleotide sequence optimized for plant expression.

Claim 57 (Original): The sequence of claim 56, wherein said optimization is the addition of a histidine tag.

Claim 58 (Original): The sequence of claim 56, wherein said optimization is the addition of an ER retention signal and histidine tag.

Claim 59 (Original): The sequence of claim 56, wherein said nucleotide sequence is selected from the group consisting of SEQ ID NO. 2 and SEQ ID NO. 7.

Claim 60 (Original): The sequence of claim 56, wherein said sequence is a canine sequence.

Claim 61 (Original): A canine GAD65 nucleotide sequence of SEQ ID NO. 4.

Claim 62 (Original): The nucleotide sequence of claim 61, wherein said sequence is further optimized for plant expression.

Claim 63 (Original): The nucleotide sequence of claim 62, wherein said optimized sequence is represented by SEQ ID NO. 5.

Claim 64 (Original): A vector for cell transformation, said vector selected from the group consisting of SEQ ID NO. 1, SEQ ID NO. 3 and SEQ ID NO. 6.

Claim 65 (Original): A vector selected from the group consisting of pDAB771; pDAB773; pDAB2407; pDAB2457; pDAB2455; pDAB2456; pDAB3736; pDAB3741; pDAB3731; pDAB3748; pDAB2453; pDAB4005; pDAB2451; and pDAB8504.

Claim 66 (Original): A composition comprising a mixture of anti-CD3 antibodies and a preparation that contains at least one autoantigen and an immunoregulatory cytokine.

Claim 67 (Original): A composition comprising a mixture of anti-CD3 antibodies and a transgenic plant material that contains at least one autoantigen and an immunoregulatory cytokine.

Claim 68 (Original): The use of a composition comprising anti-T cell antibodies, autoantigen and optional mucosal antigen in the manufacture of a medicament for the treatment of Type I diabetes in a mammal.

Claim 69 (Original): A method for the diagnosis of Type I diabetes in a mammal, the method comprising detecting in a sample from said mammal the presence of anti-GAD antibodies, such detection being an early indicator of the development or the risk of development of Type I diabetes in the mammal.

Claim 70 (Original): The method of claim 69, wherein the anti-GAD antibodies are canine antibodies.